Newborn screening for severe combined immunodeficiency: breaking the bubble

Blom, M.

Newborn screening for severe combined immunodeficiency

Breaking the bubble

Maartje Blom

Newborn screening for severe combined immunodeficiency

Breaking the bubble

Maartje Blom

Newborn screening for severe combined immunodeficiency*:*
Breaking the bubble

Cover design and layout: © evelienjagtman.com
Printing: Ridderprint | www.ridderprint.nl

ISBN: 978-94-6416-661-3

The printing of this thesis was financially supported by the Leiden University Medical Center (LUMC), the National Institute for Public Health and the Environment (RIVM), ImmunoIVD, Epimune GmbH and PerkinElmer.

Newborn screening for severe combined immunodeficiency

Breaking the bubble

Proefschrift

ter verkrijging van de graad van
doctor aan de Universiteit Leiden
op gezag van rector magnificus prof.dr.ir H. Bijl
volgens het besluit van het college voor promoties
te verdedigen op

woensdag 2 maart 2022 klokke 15:00 uur

door

Maartje Blom

Promotor
Prof. dr. A.C. Lankester

Co-promotores
Dr. M. van der Burg
Dr. R.G.M. Bredius

Promotiecommissie

Prof. dr. R. ten Cate	
Prof. dr. F.J.T. Staal	
Prof. dr. A.C. Gennery	Great North Children's Hospital, New Castle, United Kingdom
Dr. R.H. Zetterström	Karolinska Institute, Stockholm, Sweden

TABLE OF CONTENTS

CHAPTER 1

General introduction

1

GENERAL INTRODUCTION

The story of David Vetter – 'The boy in the bubble' moved a world he couldn't touch
In the early 1970s, an unusual boy captured the world. On September 21st, 1971, David Phillip Vetter was born at the Texas Children's Hospital in Houston. After 20 seconds of exposure to the world, he was placed in an isolating, sterile, plastic bubble. David was diagnosed with severe combined immunodeficiency (SCID), a hereditary immune condition preventing him from fighting off infections caused by everyday pathogens. Without a working immune system, any germ he picked up could have been lethal. At the time of his birth in 1971, a bone marrow transplant from a matched donor (HLA matched family donor) was the only possible cure for SCID, but there was no matched donor available in David's family. As David grew older and doctors continued searching for a cure, David's life in the bubble became permanent. His mother Carol Ann explained, "*There was never any plan to keep David in there – in the bubble – indefinitely. To keep a child isolated, unable to touch, or feel, or smell, or enjoy, sounds cruel, perhaps. What did they expect us to do – take David out of the bubble, which would have been certain death?*". David had grown into an adolescent without a clear road forward, but medical advances provided new hope. By 1983, a new technical approach of bone marrow transplantation had been developed with unmatched donors. David's sister Katherine donated her marrow and David received the stem cell graft. At first, the procedure seemed to work, but a dormant and undetected Epstein-Barr virus (EBV) in Katherine's marrow, triggered the growth of Burkitt's lymphoma that overwhelmed David's body. Eventually, it became necessary to remove David from the bubble for what would be the last two weeks of his life. For the first time in his life, his parents were able to hold him without a sheet of plastic between them. David died two weeks after entering a world his body could not tolerate.

David Vetter inside his sterile bubble

David (age 12) with his mother Carol-Ann

|Courtesy Baylor College of Medicine Archives|

SEVERE COMBINED IMMUNODEFICIENCY

Severe combined immunodeficiency (SCID) is the most severe form of inborn errors of immunity (IEI) characterized by the absence or dysfunction of T-lymphocytes, often accompanied by the lack of B-lymphocytes and NK-cells affecting both cellular and humoral immunity [1]. SCID is a term used to describe a disease entity caused by various genetic defects. The incidence of SCID is estimated to be in 1 in 50,000 to 100,000 births, but varies depending on geographical and ethnic background [2-4]. Infants with SCID typically appear normal at birth, but develop severe infections in the first months of life. Without curative treatment, in the form of allogeneic hematopoietic stem cell transplantation (HSCT) or in some specific forms of SCID, gene therapy (GT), affected infants die within the first year of life [5]. Early definitive treatment, before the onset of infections, results in the best outcomes [6].

Disease mechanisms and molecular causes

SCID is primarily characterized by the absence or dysfunction of T-lymphocytes affecting both cellular and humoral immunity. Even if B-lymphocytes are present, they are barely functional due to the lack of T-cell help or due to intrinsic defects in B-cell function. Several molecular defects have been identified resulting in the aberrant development or absence of naïve T-lymphocytes. The IUIS expert committee has published and updated biannually a genotypic and phenotypic classification of all IEIs [7, 8]. For SCID, more than 20 different genetic defects been described. SCID gene lists have grown and become more complex as the discovery of novel IEI disorders has been occurring at an impressive rate [9]. Types of SCID can be classified by shared pathogenesis and immunological features (Figure 1).

Defects in Cytokine Receptors and Cytokine Signaling (T-B+ SCID). The most common form of SCID is X-linked SCID, caused by mutations in the *IL2RG* gene encoding for the common γ chain (γc). This common subunit is shared by cell surface receptors for various interleukin molecules (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). IL-7 is involved in expansion of early thymocyte progenitors, whereas IL-15 plays a role in NK-cell development. Patients with X-linked SCID therefore lack both T-lymphocytes and NK-cells. The number of circulating B-lymphocytes is usually normal, but as B-cells do not undergo class switching due to lack of T-cell help, their function is impaired. X-linked SCID patients often have poor B-cell function post-HSCT, suggesting an intrinsic defect in B-cell function as well [10, 11]. The γc is bound to the intracellular tyrosine kinase Janus kinase-3 (JAK3) which is activated upon cytokine binding to the receptor and delivers γc-mediated intracellular signaling. Defects in the *JAK3*

gene result in an autosomal recessive form of SCID with a T-B+NK- phenotype similar to X-linked SCID. Mutations of the *IL7R* gene (encoding for the α chain of the IL-7 receptor) abrogate T-lymphocyte development, but do not interfere with B-cell and NK-cell development [1, 12].

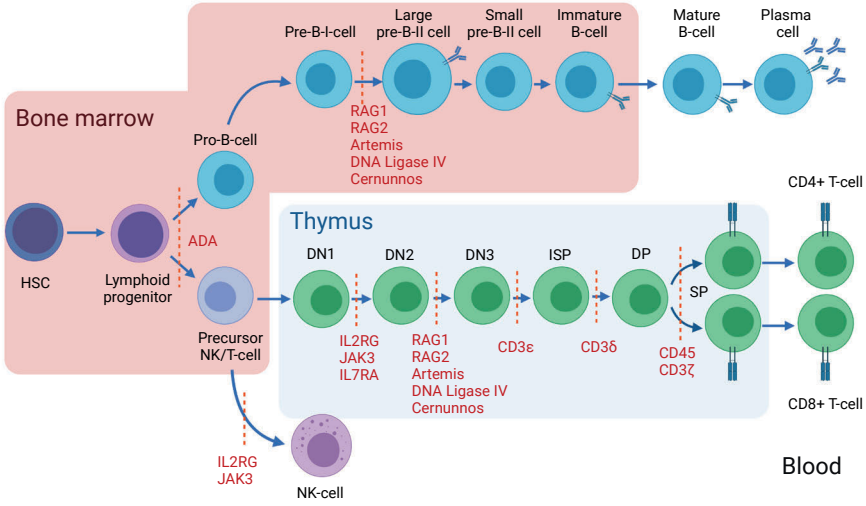

Figure 1. Schematic overview of development of human T-, B, and natural killer (NK) cells. Defects in the SCID genes with the highest incidence causing blocks in lymphoid development are indicated. Created with Biorender.com

Defective (pre-)T-cell receptor (TCR) signaling (T-B+ SCID). Defects in the key proteins involved in pre-TCR/TCR signaling can also lead to a SCID T-B+ phenotype. Mutations of the *CD3δ*, *CD3ε* and *CD3ζ* chains prevent formation of a functional CD3 complex leading to disrupted expression and signaling via the (pre)-TCR. Patients with CD3 deficiency have very low levels of mature circulating CD3+ T-cells, no CD4+ or CD8+ T-cells, and a total absence of γ/δ T-cells. T-B+ SCID can also be caused by variants in the tyrosine phosphatase *CD45* gene coding for a transmembrane protein required for T-and B-cell antigen receptor signal transduction [13].

Defects in Recombination of the Antigen Receptor Genes (T-B- SCID). A critical process during the T- and B-cell development is the somatic rearrangement of the antigen receptor genes on T- and B-cells, generating clonal diversity. *RAG1* and *RAG2* genes encode proteins that introduce DNA double-strand breaks at recombination signal sequences (RSSs), permitting V, D, and J gene rearrangements. *RAG1* or *RAG2* mutations result in a functional inability to form antigen receptors, disrupting

development of both T- and B-lymphocytes, whereas NK-cell development is not affected. Impaired V(D)J recombination may also be due to genetic defects in components of the non-homologous end joining pathway (NHEJ) such as defective DNA end-binding (DNA PKcs), DNA end-processing (Artemis/*DCLRE1C*) and DNA ligation (LIG4, XLF/Cernunnos). Genetic defects in one of the NHEJ factors are also characterized by radiosensitivity and accompanied with other manifestations such as microcephaly and facial dysmorphisms. Similar to RAG1 and RAG2 SCID, development of T- and B-lymphocytes is severely impaired in these genetic conditions while NK-cells proceed normally [14, 15].

Defects in Purine Pathway Enzymes (T-B- SCID). Autosomal recessive SCID is most commonly caused by mutations in the adenosine deaminase (*ADA*) gene leading to ADA deficiency (10% to 15% of all forms of SCID). ADA deficiency results in buildup of toxic metabolites, leading to premature lymphocyte precursor cell death. In the case of complete absence of enzymatic activity, accumulation of adenosine and deoxyadenosine will induce apoptosis, resulting in a T-B-NK- phenotype. Milder forms with residual ADA activity have been reported, leading to delayed diagnosis of immunodeficiency after several months (delayed onset) or even later occurring after two to three years (late onset). Purine nucleoside phosphorylase (PNP) is another enzyme of the salvage pathway of purine metabolism. PNP deficiency is unique among IEI as T-lymphocytes progressively decrease, while auto-immune hemolytic anemia and neurological impairment can occur as well [16, 17].

Impaired survival of lymphocyte precursors (T-B-SCID). A rare autosomal recessive form of SCID is reticular dysgenesis (RD). This rare condition is caused by mutations of the adenylate kinase 2 gene (AK2). AK2 deficiency is not only associated with blocked lymphoid differentiation, but also results in apoptosis of the myeloid precursors. Patients with RD may present with neutropenia, deafness and in some cases with anemia and thrombocytopenia [18].

Hypomorphic mutations in SCID genes. Hypomorphic mutations in several genes that cause SCID can give rise to an incomplete defect leading to a leaky SCID phenotype, a less profound combined immunodeficiency (CID) phenotype or in Omenn syndrome. Both leaky SCID and Omenn syndrome can be associated with presence of variable numbers of T-lymphocytes with poor immune function. Auto-immune manifestations are common in these patients due to inadequate control of autoreactivity and the infiltration of target tissues by activated and oligoclonal T-cells. Omenn syndrome was originally described in patients with mutations in *RAG 1* and *RAG2,* but has now been identified in a growing list of other leaky SCIDs with mutations in *Artemis*, *IL7RA*, *LIG4*, *ADA* and *IL2RG [19]*.

Clinical manifestations

Without adaptive immunity, patients with SCID are prone to severe, recurrent infections caused by both non-opportunistic and opportunistic pathogens. Patients are usually born asymptomatic, but develop life-threatening infections, failure to thrive and in some cases chronic diarrhea in the first months of life. Opportunistic infections such as *Pneumocystis jiroveci* pneumonia (PCP) and viral infections can have fatal outcomes in SCID patients. Bacterial infections are less common in part because of the presence of maternal Ig-antibodies in early infancy. With the exception of mucocutaneous candidiasis, severe invasive fungal infections are rare in SCID patients. In countries with neonatal BCG vaccination programs, Bacillus Calmette-Guerin (BCG)-vaccine-related complications may occasionally be the presenting feature in immunized SCID patients. Non-infectious clinical manifestations consist mainly of graft versus host disease (GvHD) caused by the patient's inability to reject allogenic lymphocytes acquired either from mother *in utero* or from unirradiated blood transfusion [1, 15].

Leaky SCID patients usually survive beyond 12 months of age and can present with recurrent infections and immune dysregulation including auto-immune manifestations such as auto-immune cytopenia and EBV-driven lymphoproliferative disease. It is important to consider and recognize atypical SCID presentation in children presenting beyond the first year of life [20]. Patients with Omenn syndrome can present with a progressive erythematous rash (erythroderma) which may often cause alopecia and loss of eyebrows and eyelashes. These symptoms can be present at birth but can also evolve over the first weeks of life. Lymphadenopathy, hepatosplenomegaly, high IgE levels and eosinophilia are frequent findings. Patients with Omenn syndrome often suffer from diarrhea, failure to thrive and persisting infections as seen in other forms of SCID [21].

Diagnostics

Awareness of clinical manifestations and laboratory features that indicate an underlying cellular immunodeficiency amongst primary caregivers and pediatricians is critical in the diagnostic process of SCID. Flow cytometric immunophenotyping of (naïve) T-, B-, and NK-cells is the classically recommended method in the diagnostic work-up in case of a suspicion of SCID. SCID is primarily characterized by very low or absent naïve T-cells (< 200 naïve $CD4^+$ T-cells/μL). Interpretation of flow cytometric results is more complicated in patients with Omenn syndrome or leaky SCID, as the patients can present with high numbers of oligoclonal T-cells or maternal engraftment. A detailed analysis of T-cell subsets is therefore of utmost importance. Diagnostic criteria that describe the most important features of SCID might facilitate diagnosis of SCID, helping physicians regardless of their familiarity with IEIs (Table 1) [22].

Table 1. Diagnostic criteria for typical SCID, leaky SCID and Omenn syndrome. Table adapted from the PIDTC classification, 2014 [22].

Typical SCID
Absence or very low number of T-cells (CD3 T-cells < 300/microliter), AND no or very low T-cell function (< 10% of lower limit of normal) as measured by response to phytohemagglutinin (PHA) **OR** T-cells of maternal origin present
Leaky SCID
Reduced number of CD3 T-cells • For age up to 2 years < 1000/microliter • For > 2 years up to 4 years < 800/microliter • For > 4 years < 600/microliter • Absence of maternal engraftment • 30% of lower limit of normal T-cell function (as measured by response to PHA)
Omenn syndrome
• Generalized Skin Rash • Absence of maternal engraftment. • Detectable CD3 T-cells, ≥ 300/microliter • Absent or low (up to 30% of normal) T-cell proliferation to antigens to which the patient has been expose If the proliferation to antigen was not performed, but at least 4 of the following 10 supportive criteria, at least one of which must be among those marked with an asterisk (*) below are present, the patient is eligible for the diagnosis Omenn syndrome • Hepatomegaly • Splenomegaly • Lymphadenopathy • Elevated IgE • Elevated absolute eosinophil count • Oligoclonal T-cells measured by CDR3 length or flow cytometry* • > 80% of CD3+ or CD4+ T-cells are CD45RO+ • Proliferation to PHA is reduced < 30% of lower limit of normal* • Proliferative response in mixed leukocyte reaction is reduced < 30% of lower limit of normal* • Mutation in SCID-causing gene*

In addition to flow cytometry, HIV-infections which could also cause severe recurrent infections and T-cell deficiency must be ruled out. Functional assays, assessing T-cell function can be done by *in vitro* measurement of responses to mitogens such as phytohemagglutinin (PHA). It is important to evaluate the humoral immunity by measurement of Ig levels while taking maternal transplacental antibodies into account. Every effort should be made to identify infections, and biopsy material including culture of appropriate tissue specimens and PCR may be needed to identify infecting pathogens. The definite diagnosis of SCID is ascertained by genetic analysis to identify the underlying disease-causing defect. Next generation sequencing (NGS) based on targeted panel

sequencing or whole exome sequencing (WES) with filter for SCID genes are increasingly used to identify variants in known SCID genes. WES or whole genome sequencing allow the identification of genetic defects in new IEI candidate genes [23, 24].

Treatment

Isolation and supportive care. Infants suspected of having a SCID should be placed in protective isolation with strict handwashing procedures to minimize exposure to (hospital-acquired) infections. Prophylaxis for bacterial infections and PCP should be started as soon as possible, while antifungal prophylaxis should also be considered [25]. Active infections should be treated vigorously. Discontinuation of breast feeding in CMV positive mothers is an ongoing topic of discussion, however, the risk of a neonatal CMV infection transmitted through breast milk in these severely immunocompromised newborns, may outweigh the benefit of breast-feeding [26]. Antiviral prophylaxis such as valganciclovir should be considered while awaiting maternal CMV results [27]. Blood products should be CMV-negative and irradiated to avoid the risk of transfusion GvHD. Live attenuated vaccines, such as rotavirus and varicella, should be avoided.

HSCT. HSCT has been the gold standard for treatment of SCID ever since the first stem cell transplantations in North America and Europe in 1968 [28, 29]. This lifesaving treatment reconstitutes a functional immune system by infusion of donor stem cells. Various stem cell sources can be used, including stem cells from bone marrow, mobilized peripheral blood stem cells or those harvested from umbilical cord blood. Donor types include HLA identical siblings, other matched family donors, (mis-)matched unrelated and mismatched related donors. Since the case of David Vetter in 1971, survival after HSCT has continued to improve due to refinement of HLA-tissue typing methods, improved methods of isolating $CD34^+$ hematopoietic stem cells (HSCs) and development of more effective (*ex vivo*) T-cell depletion methods. In addition, molecular detection of viral infections has enabled pre-emptive treatment of viremia and more effected treatment of transplant-related complications have led to an overall survival (OS) of 85 to 90% post HSCT [6, 30, 31]. There are a number of factors associated with better survival and outcomes after HSCT, but having an HLA-matched sibling donor and absence of active infections or organ damage prior to transplantation seem to be the most important ones [6, 32, 33]. A successful transplant procedure is lifesaving and in most cases curative with patients leading normal lives off medication, but some complications might occur. There is the risk of rejection or graft failure requiring a second transplant, in particular, when no conditioning is used. The role of chemotherapy conditioning regimens pre-HSCT is an ongoing topic of discussion. HSCT for SCID can be performed without any conditioning regimens which is associated with a lower incidence of GvHD without chemotherapy-induced toxicity [5]. However, condition regimes that contain (a certain level) of myeloablative agents are associated

with better donor myeloid engraftment and better T- and B-lymphocyte reconstitution [34]. Especially for patients with absent or non-functioning B-lymphocytes, conditioning is usually needed to acquire normal B-lymphocyte function post-HSCT [35, 36]. There are many patients after HSCT living with the effects of poor immunity or sequelae of both pre- and post-transplant complications. Approximately 25% of patients require life-long immunoglobulin replacement therapy because of the absence of donor B-lymphocyte engraftment [35]. One of the most significant adverse events of HSCT is the development of GvHD. GvHD occurs due to the recognition of host MHC antigens by donor T-cells leading to a range of symptoms and manifestations. GvHD can be categorized in acute GvHD, usually developing within three months post-HSCT and chronic GvHD. *Ex vivo* T-cell depletion of the graft, GvHD prophylactic mediation, serotherapy in the conditioning regimen and cyclophosphamide after graft infusion are strategies to prevent GvHD. Some sequelae relate to the specific genetic defect such as human papillomavirus-associated warts in patients with IL2RG/JAK3 SCID, neurodevelopmental disorders in ADA deficiency or late toxicity after HSCT with growth retardations and endocrinologic deficiencies in Artemis patients [37, 38].

Enzyme replacement therapy (ERT). For ADA deficiency, ERT with polyethylene-glycosylated ADA injections is an alternative treatment. In the short term, ERT may allow some immune reconstruction and clearance of infection. However, ERT is expensive and results in only partial immune reconstitution, therefore it is often used as a 'bridge' treatment before proceeding to definitive therapy [39].

Gene therapy (GT). While advances in HSCT have resulted in improved outcomes, the procedure is still associated with a risk of mortality and morbidity from GvHD. These severe complications mandated a search for new treatment options leading to the pursuit of genetically modified autologous hematopoietic cells transduced with a vector. GT has the potential to correct genetic defects across hematologic lineages without many of the complications of HSCT [40]. SCID is an ideal candidate due to the clear link between defined monogenetic defects and clinical phenotype and the ability to repair the defect in the immune cells by manipulating the readily accessible HSCs. In addition, as SCID patients lack T-lymphocytes, a selective growth advantage is conferred to the corrected progenitor cells if the transgene is expressed [40]. Autologous stem cells circumvent the need for a suitable matched donor and abrogate the need for immune suppression as GvHD prophylaxis [41]. GT has gone through several developmental stages with first clinical trials with retroviral vectors for X-linked SCID and ADA-SCID dating back to the late 90 [42-45]. However, the use of γ-retroviral vectors was associated with severe complications such as vector-related leukemia and myelodysplastic events caused by insertional oncogenesis [46, 47]. Since then, safer GT approaches have been developed

including self-inactivating (SIN) γ-retroviral and lentiviral vectors which have substantially less transactivation potential [48, 49]. These advances have even led to the marketing approval of a GT product in Europe for ADA-SCID patients who lack a suitable donor for HSCT [50]. GT has significantly improved over the last two decades. The infrastructure to manufacture and deliver cellular therapies advances and an increasing number of clinical trials report high efficacy and excellent safety. While alloHSCT still remains the first choice treatment for many SCID patients to offer proven long term cure, the development of GT may offer a safe, effective, definitive therapy in the future that diminishes the immunological complications of HSCT

NEWBORN SCREENING FOR SCID

Importance of an early diagnosis

The importance of an early diagnosis is demonstrated by studies showing improved survival of SCID patients diagnosed at birth due to a positive family history (OS 85-90%) compared to the first presenting family members (OS 40-42%) [51, 52]. These observed differences were irrespective of conditioning regimen, donor source, or underlying (genetic) diagnosis suggesting the relation between improved survival and early diagnosis. In addition, retrospective multi-center studies in larger SCID patients' cohorts have shown that patient outcomes are significantly improved when curative therapy with HSCT is performed before the age of 3.5 months and/or prior to the onset of severe and debilitating infections [32]. Survival rates were adversely impacted by active infection pre-transplantation; 81% for patients with active infection at the time of transplantation versus 95% in infection free patients [6]. These findings suggest that an early diagnosis and the prevention of infections are predominant determinants of a good transplantation outcome.

Many SCID cases are sporadic, with no positive family history leading to prompt early diagnosis. Infants with SCID appear healthy at birth and are diagnosed after frequent medical encounters for recurrent and persistent (opportunistic) infections and/or failure to thrive. These nonspecific disease manifestations can lead to delay in recognition of the underlying disease and subsequently to delay in treatment. Realistically, an early diagnosis prior to the development of life-threatening complications is only achievable by early identification of infants with SCID through newborn screening (NBS) programs.

How can you screen for SCID? T-cell receptor excision circles

Several screening strategies have been proposed to identify patients with SCID directly after birth. A complete blood count (CBC) was suggested to detect T-cell lymphopenia, but this simple laboratory test lacked sensitivity as patients with present

B-lymphocytes, maternal engraftment or oligoclonal expansion would be missed [53]. The same was the case for protein immunoassays on dried blood spots for T-cell specific markers such as CD3 [54]. Subsequently, flow cytometry to determine T-cell populations in cord blood was also considered but proved to be too time-consuming and expensive for a population screening test [55]. There was need for an extremely sensitive and specific biomarker that could identity T-cell lymphopenia in dried blood spots (DBS) while avoiding excessive costs and anxiety associated with false-positive screen results [56].

V(D)J recombination of the TCR loci is the process whereby a diverse repertoire of antigen receptors is generated. In each T-cell randomly chosen combinations of variable (V), diversity (D) and joining (J) segments are formed to synthesize a unique rearrangement in each cell. Only T-cell progenitors with in-frame rearranged locus are selected to survive and mature. The excised DNA fragments that are not destined to be incorporated into the mature TCR locus can be joined at their ends to form a great variety of circular DNA byproducts, called T-cell receptor excision circles (TRECs). Precursor T-cells in the thymus first start to rearrange their *TCRD* and *TCRG* genes. When this leads to a functional receptor, the cell exits the thymus as TCRγδ+ T-cell. Most cells, however, do not form a functional γδ TCR and start rearranging their *TCRB* and *TCRA* genes. *TCRD* deleting rearrangements therefore exist for only a short period during thymocyte differentiation [25]. The δREC–ψJα rearrangement in the *TCRA* locus excising the *TCRD* gene is initiated after unsuccessful generation of a γδ TCR. It is estimated that 70-80% of the thymocytes that ultimately express αβ TCR form a specific circular DNA TREC in this process: the δRec-ψJα signal joint TREC [57] (Figure 2). The δRec-ψJα coding joint might still be present on the nonfunctional *TCRAD* allele and by subsequent Vα–Jα rearrangements, the δREC–ψJα coding joint will be removed and placed on a novel excision circle [58]. Quantitative PCR amplification across the joined ends of the δRec-ψJα TREC reflects the number of recently formed T-cells in peripheral blood.

TRECs were found to be unique to naïve αβ T-cell and memory T-cells lack the δRec-ψJα signal joint mentioned above. In addition, TRECs were considered to be an ideal marker for naïve T-cell production as they were noted to be stable and remained in the cytoplasm of the T-cells, not replicating during mitosis. As a result, TRECs become diluted when the T-cell population expands through cell division [60]. In 2005, the first application of quantitative PCR for TREC detection as a large-scale population screening method for SCID was described [61]. SCID became the first immune disorder in the NBS program and at the same time the TREC assay became the first high-throughput DNA-based NBS test.

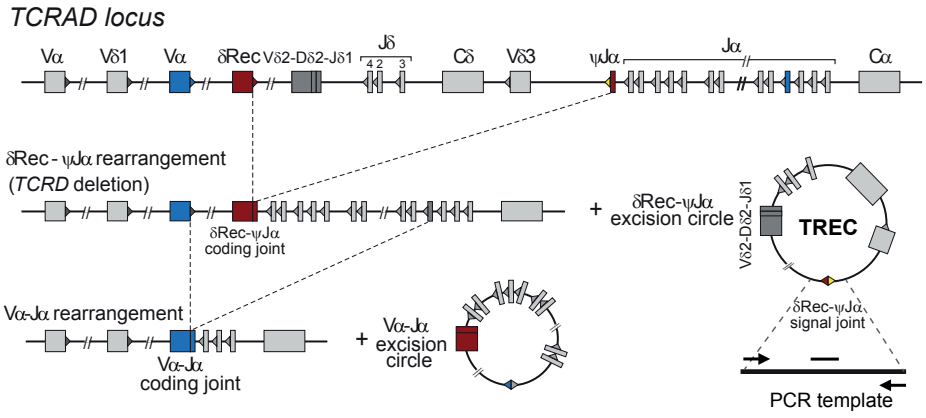

Figure 2. TRECs are stable, circular fragments of DNA formed during by excisional rearrangements of the TCR genes. During the δREC–ψJα rearrangement in the *TCRA* locus, the *TCRD* gene is excised and the δRec-ψJα signal joint TREC is formed. This specific TREC is produced by 70-80% of the αβ T-cells. With quantitative PCR amplification across the joined ends of the δRec-ψJα TREC the number of recently formed T-cells in peripheral blood can be determined [59].

Follow-up after abnormal TREC values

Most screening tests, including the TREC assay, are not designed to establish a diagnosis, but rather to signal the potential for a serious condition for which specific follow-up is required [62]. Low TREC levels indicate that a T-cell developmental problem might be present, but referral to the pediatric-immunologist is needed to confirm T-cell lymphopenia and to identify the underlying cause [63, 64]. An important part of this initial evaluation is a thorough family history and physical examination. A maternal history can reveal factors that influence T-cell numbers, such as immunosuppressive medication. A family history of unusual or fatal infectious events or unexplained infant death is important, particularly in consanguineous families. Recognizing dysmorphic features is key in physical examination.

NBS for SCID introduced clinical immunologists to diagnostic testing of apparently healthy newborns without any medical history of infections or other manifestations. Confirmatory testing strategies after an abnormal TREC value might differ between individual screening programs, but flow cytometry to enumerate CD3+ T-cells, CD4+ and CD8+ T-cells, CD56/16 NK-cells and CD19 B-cells and T-cell subsets CD45RA/CD45RO (%) naive T-cells is the cornerstone [65]. If naïve T-cells are low (< 200 cells/μl) SCID might be suspected and additional SCID diagnostics and management will be initiated. Even though TREC-based NBS programs are primarily aimed at the detection of SCID, low TRECs can be identified in a range of other conditions associated with impaired T-cell production or loss of T-cells from the peripheral circulation. These

non-SCID conditions can be referred to as incidental findings, secondary findings or even primary/secondary targets depending on the NBS program. For infants with low T-cells (300-1500 cells/μL), reduced but present naïve CD4 T-cells and no maternal cells, initial immune evaluation might be similar to that for SCID, but hospitalization may not be required if immunodeficiency is not profound. Specific diagnostic testing and management of these conditions will depend on the comorbidities.

Non-SCID cases identified via NBS for SCID

Low TREC levels can be identified in other forms of IEI such as less profound combined immunodeficiencies classified by the IUIS [8]. Newborns with a recognized genetic syndrome that include low T-cell numbers within its spectrum of clinical findings can also present with low TREC numbers. Examples are newborns with 22q11.2 deletion syndrome (DiGeorge syndrome), CHARGE-syndrome, trisomy 21, ataxia telangiectasia, trisomy 18 and Jacobsen syndrome. TRECs and T-cell numbers can also reversibly reduced due to secondary causes such as congenital malformations (e.g. cardiac or gastrointestinal anomalies), or disease processes without an intrinsic defect in production of circulating cells (e.g. loss into third space in hydrops or chylothorax or vascular leakages in sepsis) [2, 66]. Maternal immunosuppressant use can also be a cause of transient neonatal T-cell lymphopenia [67, 68]. In these cases, T-cell lymphopenia usually resolves once excess T-cell losses or suppression of T-cell maturation has been abrogated. In newborns with idiopathic T-cell lymphopenia, TRECs and T-cells might be low without an identified underlying cause, even after immunologic and comprehensive genetic evaluation. For infants with T-cell lymphopenia, longitudinal immunological evaluation is important to determine if the T-cell lymphopenia is transient [69].

Not all serious disorders affecting T-cell function can be identified via TREC screening. Combined immunodeficiencies such as ζ-associated protein of 70kDa (ZAP-70) deficiency or MHC class I and II gene expression deficiency, have severely impaired T-cell function but can have normal TREC levels as T-cell development is intact beyond the point of TCR gene recombination [70].

Infants with preterm birth (gestational age <37 weeks) and/or low birth weight are a disproportionate source of abnormal TREC results [50]. T-cell lymphopenia in these infants is depending on the degree of thymic maturity, although T-cells are not functionally impaired, and T-cell numbers usually normalize with increasing gestational age. Many NBS programs have incorporated adaptations in their screening algorithms (different cut-off values or second NBS cards) for preterm infants with low TREC levels to avoid high referral rates.

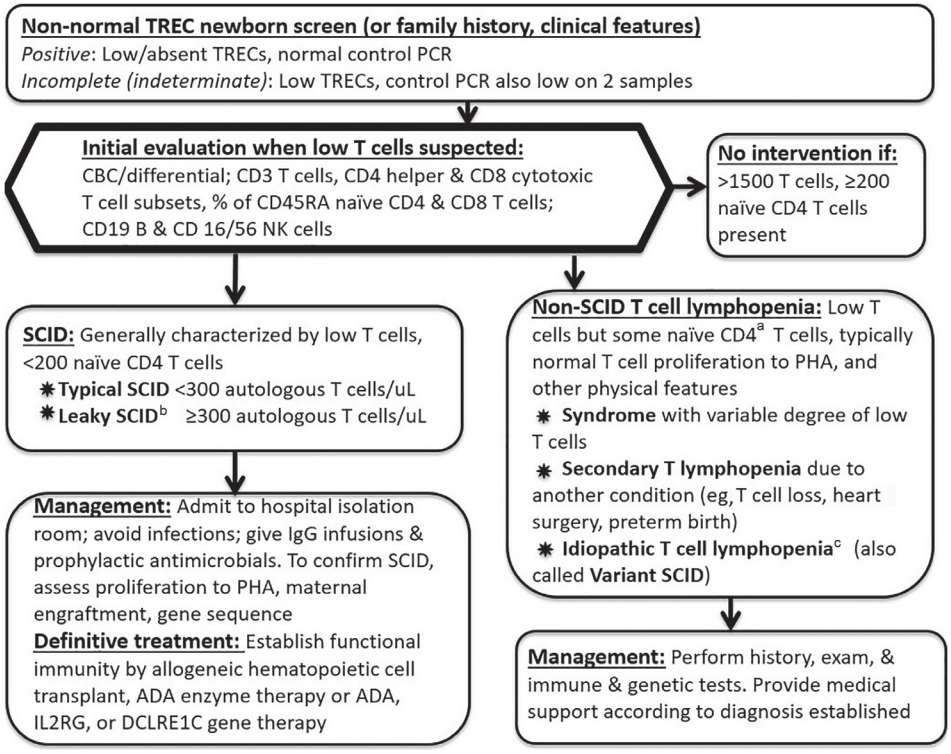

Figure 3. Example of a follow-up scheme after an abnormal TREC value in NBS for SCID. Figure from Dorsey *et al.* 2017 [65].

Finally, in the case of an abnormal TREC value, but normal levels of T-cells (> 1500/µL and > 200 naïve/µl) no further immunological work-up is required within the SCID screening context [65]. In these cases, TRECs could have been low at the time of the heel prick due to transient T-cell lymphopenia that resolved in the first weeks up to referral. TRECs could also be low due to technical test errors leading to false-positive results. Uniform follow-up protocols are required for a prompt and consistent approach to a definitive diagnosis and can provide guidance for pediatrician-immunologists when dealing with these non-SCID cases identified via NBS for SCID (Figure 3).

IMPLEMENTATION OF SCID IN NBS PROGRAMS

General background information NBS programs

The primary aim of NBS programs is to identify potentially fatal or disabling conditions in pre-symptomatic newborns for which timely intervention is available and critical to improve the outcome. These conditions might not be evident at birth, but if left undiagnosed and untreated could have fatal or severe developmental consequences for the child. With early detection and early intervention, morbidity and mortality can be reduced. In addition to individual health benefits, NBS also aims to minimize negative societal and economic impacts of life-threatening diseases [71]. Since the initiation of NBS in the 1960s with screening for phenylketonuria (PKU), innovations have led to the gradual expansion of screened conditions in NBS panels. The introduction of tandem mass spectrometry led to a boost in the late 1990s allowing the simultaneous biochemical analysis for a significant number of inborn errors of metabolism (IEM). The availability of tandem mass spectrometry led to test-driven expansions in NBS programs worldwide, with some NBS programs currently screening for more than fifty conditions [72, 73].

Most programs are structured to screen for a number of core disorders, along with secondary target disorders. The spectrum of disorders included in NBS programs greatly varies between countries. National health care politics, healthcare structures, input from patient advocacy groups and different interpretations of screening criteria have led to differences in panels of screened conditions [73, 74]. The recommendations of Wilson and Jungner (1968) for populated-based disease screening are the backbone of the screening policy [75]. Since their publication in 1968, these criteria have provided a framework against which conditions can be assessed for their suitability for screening, being of aid in decision making with regard to inclusion of new disease candidates in NBS programs. The criteria have been refined in 2008 by the WHO due to the growing interest in genetic screening and changing demands of modern times (Table 2) [76].

Table 2. Criteria used for inclusion of new conditions in NBS programs

Original Wilson and Jungner criteria (1968) [75]	**Additional WHO-criteria (2008) [76]**
1. The condition sought should be an important health problem	1. The screening program should respond to a recognized need.
2. There should be an accepted treatment for patients with recognized disease	2. The objectives of screening should be defined at the outset
3. Facilities for diagnosis and treatment should be available	3. There should be a defined target population
4. There should be a recognizable latent or early symptomatic stage	4. There should be scientific evidence of screening program effectiveness
5. There should be a suitable test or examination	5. The program should integrate education, testing, clinical services and program management
6. The test should be acceptable to the population	6. There should be quality assurance, with mechanisms to minimize potential risks of screening
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood	7. The program should ensure informed choice, confidentiality and respect for autonomy
8. There should be an agreed policy on whom to treat as patients	8. The program should promote equity and access to screening for the entire target population
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole	9. Program evaluation should be planned from the outset
10. Case-finding should be a continuing process and not a “once and for all” project	10. The overall benefits of screening should outweigh the harm

Organization of a NBS program

As previously mentioned, most screening tests are not designed to establish a diagnosis, but rather to signal the potential for a serious condition for which specific follow-up is required. Screening should therefore be thought of as an integrated program or a system and not merely a test [62]. Organization of population screening programs are complex due to the involvement of many stakeholders. It is important to realize that countries have their own distinguished manner of organizing their health care system and this also applies to the NBS system.

The NBS system includes preanalytical, testing, and postanalytical phases. The preanalytical phase includes collection of demographic data, blood sampling and shipment of NBS cards. In the preanalytical phase communication is key. Most countries verbally inform parents with the aid of written brochures or websites prior to sample collection. Information is usually provided by a neonatologist, midwife or nurse [77]. In Europe, sample collection is usually performed between 48-72 hours after birth, while NBS programs in the US have an earlier sampling window of 24-48 hours after birth. These differences are mostly due to differences in the organization of the NBS program and maternity care. Some countries perform the heel prick in the hospital before discharge, while in other countries sample collection is done by midwifes or screeners at home [78]. The testing phase usually occurs at designated department of health laboratories and includes samples preparation, test conduction, results interpretation, and report issuing. The final and most important phase is the postanalytical phase where abnormal NBS results requiring further testing are communicated and confirmed, treatment is initiated, and long-term follow-up is monitored [71]. Typically, the laboratory reports abnormal NBS results to the primary health care provider, who will subsequently notify the family and refer the infant to the pediatric-specialist. In Europe, screening results are primarily confirmed in specialized centers. Several countries make all screening results available to parents either online, by mail or by post. Other countries only inform parents if an action is required, such as a referral or a request for a second sample [79]. The Dutch NBS structure is depicted in Figure 4. Key aspects for the success of NBS programs are timelines of sample transport, quality assurance for performed tests, good and clear communication to parents, easy access to health care and continuous program evaluation. Ongoing tracking of test performance and outcomes must be part of every screening program, with regular communication and adjustments to improve sensitivity, specificity, turnaround times, follow-up care, cost effectiveness and outcomes. Sharing of information at every level makes the program efficient, but also affords opportunities for new insights [62].

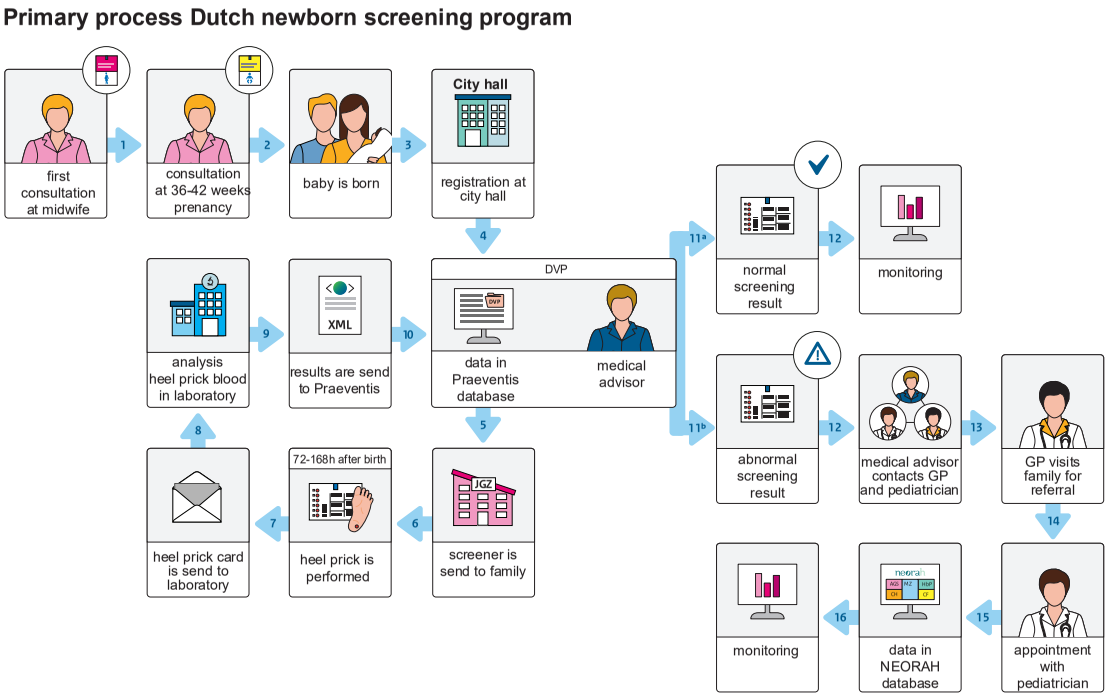

Figure 4. Primary process Dutch newborn screening program. Figure available via RIVM-website. Expecting parents will receive information about the NBS program during the first and second consultation with the midwife (1-2). During registration at city hall, an information brochure will be handed out as well (3). The screening organizations (JGZ) will be informed about the registration of the newborn, after which screeners will visit the family to perform the heel prick (6). The heel prick card is sent by post to one of the five screening laboratories (7). The heel prick cards are then analyzed, and the results are registered in the national monitoring database Praeventis (8-9). Abnormal results are forwarded to the general practitioner (GP) and pediatrician by the medical advisor (12-13). Medical advisors coordinate logistics of the referral procedure. GPs will visit the family to inform them about the referral after which the family will visit the pediatrician for follow-up diagnostics (14).

Cultural differences and expansion of NBS programs

While significant treatment- and test-driven expansions are seen in several NBS programs worldwide, other NBS programs expand at a slower rate. This illustrates that even though screening tests and treatments are available, the local context will determine the NBS program put in place [72]. The United States wields a more liberal approach when it comes to expansion of their NBS program with an increasing number of disorders being recommended for inclusion. As of July 2018, the Recommended Uniform Screening Panel (RUSP) includes 35 core conditions and 26 secondary targets [80]. In the US, public opinions can greatly influence NBS policies. Carol Ann, mother of David Vetter together with the Immune Deficiency Foundation (IDF) launched a successful advocating campaign for NBS for SCID. However, problems have also arisen from parent group advocacy campaigns pressuring individual states to screen for specific, non-recommended disorders [81-83]. Europe has a more conservative and heterogenous approach when it comes to population screening programs [73]. Healthcare has always been left to the own responsibility of the member states (principle of subsidiarity) allowing each country to make its own decision with regard to conditions that should be included in NBS programs [78]. Unlike the US where public opinions are more likely to influence NBS policies, advocacy efforts concerning health policy are limited [79]. European funding for NBS is typically organized by national health care services or health insurances, making NBS free of charge for parents. This often results in complex, time-consuming governmental financial decisions when expansion and inclusions of new conditions is considered [84].

In the past years, changes in understanding of conditions, technological developments and new treatments, have fueled the expansion of NBS. Some NBS programs have developed from programs that screen for a small number of conditions to complex programs sometimes including over 50 conditions. In the genomic era, further expansion of NBS programs will lead to new technical, clinical, ethical, and societal challenges accompanied by DNA-based screening [85].

NBS for SCID pilot programs and implementation in other countries

NBS programs are a complex, multi-faceted system and introduction of a new condition can lead to disruption if all steps of the public health policy cycle are not carefully considered. While the central idea of early detection of a disorder to facilitate treatment is simple, successful implementation of NBS for a disorder is something else. Pilot studies provide the opportunity to add new conditions and evaluate feasibility and disparities before disruptions of the program can occur. In addition, pilot studies are vital to the development of a strong evidence base to support decision-making regarding the addition of new conditions.

Specifically for SCID, pilot studies were of great aid when introducing DNA analysis as a primary screening modality in NBS laboratories. In addition, as SCID is the first immunodeficiency disorder added to the NBS program, pilot studies have helped with the gradual introducing pediatric-immunologists and clinical immunologists to the field of NBS. Clinical immunologists were less familiar with pre-symptomatic apparently healthy newborns, secondary findings and false-positive referrals. As screening is imposed upon an entire population with the goal of advancing public health, it is important to appreciate the differences between population-based screening programs versus clinical care [62]. Pilot studies and international shared learning have helped clinical immunology community with uniform follow-up protocols for a prompt and consistent approach to a definitive diagnosis.

First pilot studies for NBS for SCID were performed in the US almost a decade ago. The first state-wide SCID screening pilot study was initiated in Wisconsin in 2008 [86], with subsequently implementation of NBS for SCID in Massachusetts, Louisiana, and New York in 2009, and California, Texas, and Pennsylvania in 2010 [2]. In 2010, SCID was added to the RUSP which resulted in an acceleration in the number of states screening for SCID over the following years. By the end of 2018, NBS for SCID had been adopted by public health programs in all 50 states, the Navajo Nation, and Puerto Rico [87]. Pilot and proof-of-principle studies in Europe followed some years later in Sweden, the UK, France, the Netherlands and Spain [88-94]. Multiple nations around the world have instituted population-wide NBS for SCID, including New Zealand, Taiwan, Israel, Denmark, Sweden, Norway, Germany, Iceland and Switzerland, whereas others offer SCID screening in limited areas or have published pre-implementation analyses and pilot studies (Figure 5) [95-99].

In 2015, the Health Council of the Netherlands published the report '*Neonatal Screening, New Recommendations*' stating that SCID should be included in the Dutch NBS program [100]. The Committee believed that NBS for SCID would prevent significant, irreversible damage and yield substantial health gains for the affected child, while the disadvantage of unavoidable secondary findings did not outweigh the advantage of improved treatment by early diagnosis. The Dutch Ministry of Health adopted the advice and recommended an implementation pilot study including an exact cost-benefit analysis prior to national implementation. The pilot study would not focus on whether the TREC assay was a suitable method for the detection of SCID, as the effectiveness of NBS for SCID had already been proven in other screening programs abroad. However, as NBS for SCID is executed with a new, relatively expensive assay for the screening laboratory, an implementation pilot study was deemed instrumental for successful implementation.

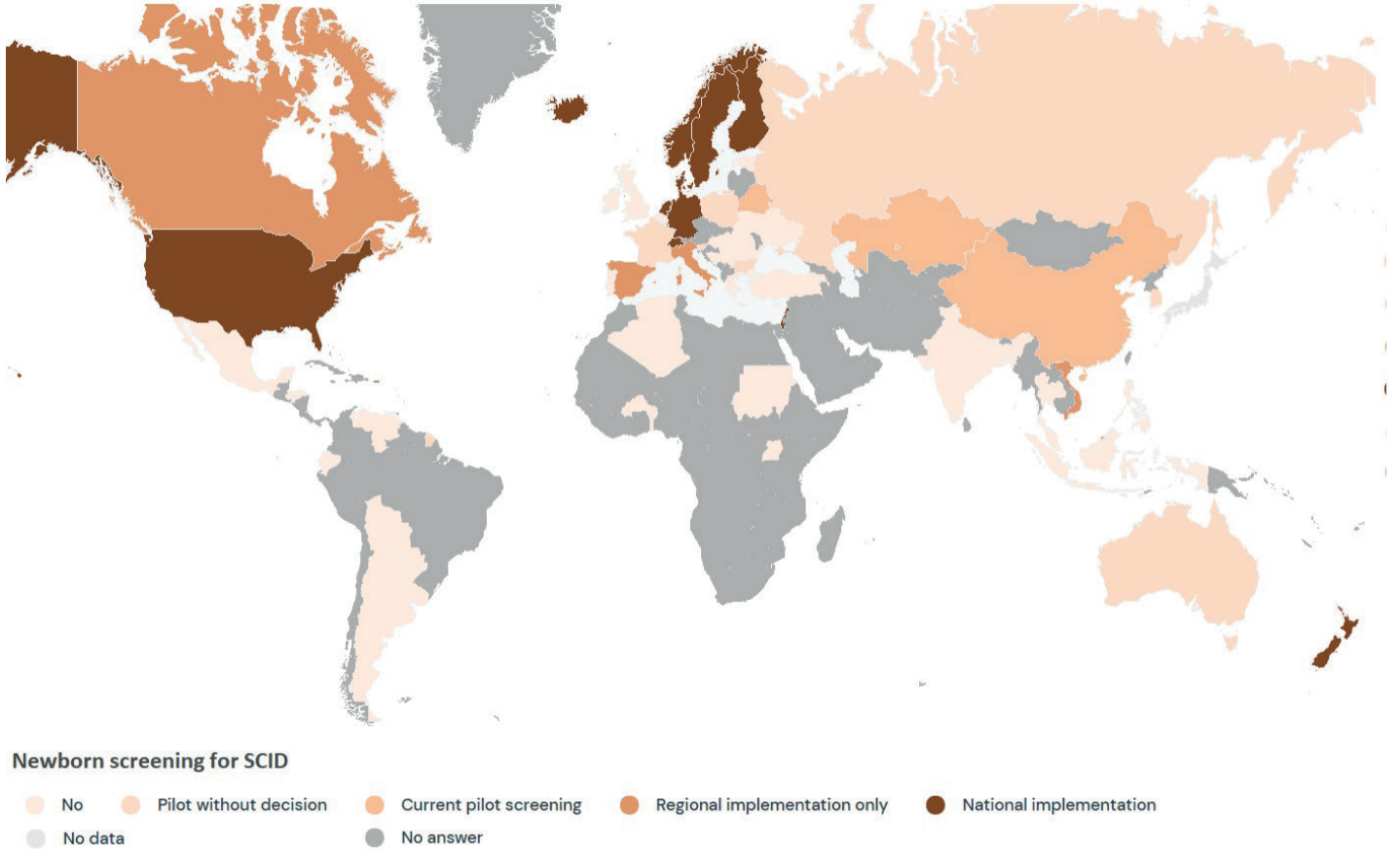

Figure 5. Overview of worldwide implementation of NBS for SCID in different countries. Figure adapted from PID Life Index (IPOPI).

AIM OF THIS THESIS

Introducing a new disorder into a screening program is a multifaceted process that needs to be carefully done without disruption of the program. This thesis therefore aimed to evaluate the many aspects that are associated with NBS for SCID, assessing feasibility and disparities prior to national implementation. NBS for SCID based on TREC detection has been implemented in many countries, with initial pilot studies dating back to 2008. The aim of this thesis was therefore not to prove the effectiveness of TREC quantification for the detection of SCID positive cases, but to obtain knowledge about practical implications, test qualities, costs and ethical and social implications of NBS for SCID. Practical implications included test modalities as NBS for SCID is associated with a new screening method, while also covering diagnostic and clinical follow-up aspects including unexpected screening outcomes and secondary findings. With a concise cost-effectiveness analysis, this thesis tried to provide an overview of costs and benefits associated with NBS for SCID, aiding in the final decisions to include SCID in the NBS program. Unique to this thesis was the inclusion of societal and ethical implications of NBS for SCID, aspects that had never been studied before. By assessing the perspectives of parents as key stakeholders in NBS, potential benefits and harms of NBS for newborns and their families could be identified. Moreover, societal acceptance is a major criterion when introducing new disorders in NBS programs and as parents are important stakeholders, their support is paramount. The ultimate aim of this thesis was to enable a flawless implementation of NBS for SCID in the Netherlands, while providing valuable recommendations for other countries that are considering SCID screening and for countries that want to optimize their implemented NBS SCID program. NBS for SCID in the Netherlands will contribute to improved outcomes for future SCID patients after HSCT: "*helping to break the protective bubble in the best possible way*".

THESIS OUTLINE

This thesis will address the many aspects of NBS for SCID from preparatory steps to pilot study to optimizing after implementation. **Chapter 2** focuses on the first preparatory steps by exploring test modalities and evaluating a commercially available TREC assay in the NBS laboratory. In **Chapter 3**, the structure of the Dutch NBS program is further specified, while different aspects needed for a pilot study are assessed and results of a comparison study between test methods are discussed. All preparatory steps led to a prospective implementation pilot called the SONNET-study (SCID screening Onderzoek in Nederland met TRECs) of which the results are discussed in **Chapter 4.** This pilot study did not only focus on the technical aspects of NBS for SCID, but also evaluated the perspectives of parents as public uptake and parental acceptance of a test method are not guaranteed. **Chapter 5** explores different second tier test options and screening strategies showing that even after implementation, NBS programs should continue to optimize their programs aiming for the highest sensitivity while limiting the number of false-positive referrals. Some outcomes of NBS are unanticipated as showed in the case report of **Chapter 6** in which newborns with abnormal NBS SCID results and profound T-cell lymphopenia due to maternal immunosuppressant use are presented. More ethical aspects are addressed as this thesis delves deeper into the dilemma of an early diagnosis of the incurable condition ataxia telangiectasia (A-T) as a secondary finding of NBS for SCID. Parents of children with A-T provide their opinion on this quandary in **Chapter 7,** while the perspectives of parents of healthy newborns are presented in **Chapter 8**. Cost-effectiveness is key when adding new conditions to a NBS program and **Chapter 9** provides a cost-effectiveness analysis for NBS for SCID in Netherlands based on real-life data from the SONNET-study. In **Chapter 10** recommendations are provided for uniform definitions of screening terminology and case definitions after follow-up in NBS for SCID. These guidelines will unite the NBS community and the clinical immunological community by bridging the gaps in language and perspective between these disciplines. Expansion of NBS with new disorders is driven by development of new test modalities and treatment options, therefore **Chapter 11** will discuss the future perspectives on NBS for SCID and other IEI that could benefit from an early diagnosis and intervention. This thesis ends with a general discussion describing new points of debate, recommendations and future directions coupled with my personal perspective in **Chapter 12** and a summary of all work presented in this thesis in **Chapter 13.**

REFERENCES

1. Fischer, A., Severe combined immunodeficiencies (SCID). Clinical & Experimental Immunology, 2000. 122(2): p. 143-149.
2. Kwan, A., et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama, 2014. 312(7): p. 729-38.
3. de Pagter, A.P., et al., Overview of 15-year severe combined immunodeficiency in the Netherlands: towards newborn blood spot screening. Eur J Pediatr, 2015. 174(9): p. 1183-8.
4. Al-Muhsen, S. and Z. Alsum, Primary immunodeficiency diseases in the Middle East. Ann N Y Acad Sci, 2012. 1250: p. 56-61.
5. Haddad, E. and M. Hoenig, Hematopoietic Stem Cell Transplantation for Severe Combined Immunodeficiency (SCID). Front Pediatr, 2019. 7: p. 481.
6. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
7. Tangye, S.G., et al., Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol, 2020. 40(1): p. 24-64.
8. Bousfiha, A., et al., Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. Journal of Clinical Immunology, 2020. 40(1): p. 66-81.
9. Tangye, S.G., et al., The Ever-Increasing Array of Novel Inborn Errors of Immunity: an Interim Update by the IUIS Committee. J Clin Immunol, 2021. 41(3): p. 666-679.
10. White, H., et al., Intrinsic defects of B cell function in X-linked severe combined immunodeficiency. Eur J Immunol, 2000. 30(3): p. 732-7.
11. Miggelbrink, A.M., et al., B-cell differentiation and IL-21 response in IL2RG/JAK3 SCID patients after hematopoietic stem cell transplantation. Blood, 2018. 131(26): p. 2967-2977.
12. Buckley, R.H., Molecular Defects in Human Severe Combined Immunodeficiency and Approaches to Immune Reconstitution. Annual Review of Immunology, 2004. 22(1): p. 625-655.
13. Kung, C., et al., Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. Nat Med, 2000. 6(3): p. 343-5.
14. Notarangelo, L.D., Primary immunodeficiencies. Journal of Allergy and Clinical Immunology, 2010. 125(2, Supplement 2): p. S182-S194.
15. van der Burg, M. and A.R. Gennery, Educational paper. The expanding clinical and immunological spectrum of severe combined immunodeficiency. European journal of pediatrics, 2011. 170(5): p. 561-571.
16. Nyhan, W.L., Disorders of purine and pyrimidine metabolism. Molecular Genetics and Metabolism, 2005. 86(1): p. 25-33.
17. Gaspar, H.B., et al., How I treat ADA deficiency. Blood, 2009. 114(17): p. 3524-32.
18. Pannicke, U., et al., Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. Nat Genet, 2009. 41(1): p. 101-5.
19. Delmonte, O.M., A. Villa, and L.D. Notarangelo, Immune dysregulation in patients with RAG deficiency and other forms of combined immune deficiency. Blood, 2020. 135(9): p. 610-619.
20. Reeve, L., et al., Do not let them slip through the net: Catching a case of leaky severe combined immunodeficiency. J Paediatr Child Health, 2020. 56(5): p. 809-811.

21. Villa, A., L.D. Notarangelo, and C.M. Roifman, Omenn syndrome: inflammation in leaky severe combined immunodeficiency. J Allergy Clin Immunol, 2008. 122(6): p. 1082-6.
22. Shearer, W.T., et al., Establishing diagnostic criteria for severe combined immunodeficiency disease (SCID), leaky SCID, and Omenn syndrome: the Primary Immune Deficiency Treatment Consortium experience. The Journal of allergy and clinical immunology, 2014. 133(4): p. 1092-1098.
23. Seleman, M., et al., Uses of Next-Generation Sequencing Technologies for the Diagnosis of Primary Immunodeficiencies. Frontiers in Immunology, 2017. 8(847).
24. Mousallem, T., et al., Clinical application of whole-genome sequencing in patients with primary immunodeficiency. The Journal of allergy and clinical immunology, 2015. 136(2): p. 476-9.e6.
25. Griffith, L.M., et al., Improving cellular therapy for primary immune deficiency diseases: Recognition, diagnosis, and management. Journal of Allergy and Clinical Immunology, 2009. 124(6): p. 1152-1160.e12.
26. Kelty, W.J., et al., The role of breast-feeding in cytomegalovirus transmission and hematopoietic stem cell transplant outcomes in infants with severe combined immunodeficiency. The Journal of Allergy and Clinical Immunology: In Practice, 2019. 7(8): p. 2863-2865.e3.
27. Dorsey, M.J., et al., Infections in Infants with SCID: Isolation, Infection Screening, and Prophylaxis in PIDTC Centers. Journal of Clinical Immunology, 2021. 41(1): p. 38-50.
28. Gatti, R.A., et al., Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet, 1968. 2(7583): p. 1366-9.
29. De Koning, J., et al., Transplantation of bone-marrow cells and fetal thymus in an infant with lymphopenic immunological deficiency. Lancet, 1969. 1(7608): p. 1223-7.
30. Griffith, L.M., et al., Allogeneic hematopoietic cell transplantation for primary immune deficiency diseases: current status and critical needs. The Journal of allergy and clinical immunology, 2008. 122(6): p. 1087-1096.
31. Dvorak, C.C., et al., The natural history of children with severe combined immunodeficiency: baseline features of the first fifty patients of the primary immune deficiency treatment consortium prospective study 6901. J Clin Immunol, 2013. 33(7): p. 1156-64.
32. Pai, S.-Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. The New England journal of medicine, 2014. 371(5): p. 434-446.
33. Cavazzana-Calvo, M., et al., Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. Blood, 2007. 109(10): p. 4575-81.
34. Shaw, P., et al., Conditioning Perspectives for Primary Immunodeficiency Stem Cell Transplants. Front Pediatr, 2019. 7: p. 434.
35. Haddad, E., S. Leroy, and R.H. Buckley, B-cell reconstitution for SCID: should a conditioning regimen be used in SCID treatment? J Allergy Clin Immunol, 2013. 131(4): p. 994-1000.
36. Abd Hamid, I.J., et al., Long-term outcome of hematopoietic stem cell transplantation for IL2RG/JAK3 SCID: a cohort report. Blood, 2017. 129(15): p. 2198-2201.
37. Gennery, A.R., et al., Long Term Outcome and Immune Function After Hematopoietic Stem Cell Transplantation for Primary Immunodeficiency. Frontiers in pediatrics, 2019. 7: p. 381-381.
38. Schuetz, C., et al., SCID patients with ARTEMIS vs RAG deficiencies following HCT: increased risk of late toxicity in ARTEMIS-deficient SCID. Blood, 2014. 123(2): p. 281-9.

39. Hershfield, M.S., Enzyme replacement therapy of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase (PEG-ADA). Immunodeficiency, 1993. 4(1-4): p. 93-7.
40. Fox, T.A. and C. Booth, Gene therapy for primary immunodeficiencies. British Journal of Haematology, 2021. 193(6): p. 1044-1059.
41. Kohn, D.B. and C.Y. Kuo, New frontiers in the therapy of primary immunodeficiency: From gene addition to gene editing. J Allergy Clin Immunol, 2017. 139(3): p. 726-732.
42. Gaspar, H.B., et al., Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet, 2004. 364(9452): p. 2181-7.
43. Cavazzana-Calvo, M., et al., Gene therapy for severe combined immunodeficiency. Annu Rev Med, 2005. 56: p. 585-602.
44. Gaspar, H.B., et al., Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. Mol Ther, 2006. 14(4): p. 505-13.
45. Aiuti, A., et al., Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science, 2002. 296(5577): p. 2410-3.
46. Howe, S.J., et al., Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. The Journal of Clinical Investigation, 2008. 118(9): p. 3143-3150.
47. Hacein-Bey-Abina, S., et al., Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. The Journal of Clinical Investigation, 2008. 118(9): p. 3132-3142.
48. Kohn, D.B., et al., Autologous Ex Vivo Lentiviral Gene Therapy for Adenosine Deaminase Deficiency. New England Journal of Medicine, 2021. 384(21): p. 2002-2013.
49. Kuo, C.Y. and D.B. Kohn, Gene Therapy for the Treatment of Primary Immune Deficiencies. Curr Allergy Asthma Rep, 2016. 16(5): p. 39.
50. Aiuti, A., M.G. Roncarolo, and L. Naldini, Gene therapy for ADA-SCID, the first marketing approval of an ex vivo gene therapy in Europe: paving the road for the next generation of advanced therapy medicinal products. EMBO Mol Med, 2017. 9(6): p. 737-740.
51. Brown, L., et al., Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. Blood, 2011. 117(11): p. 3243-3246.
52. Chan, A., et al., Early vs. delayed diagnosis of severe combined immunodeficiency: a family perspective survey. Clinical immunology (Orlando, Fla.), 2011. 138(1): p. 3-8.
53. Buckley, R.H., et al., Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. J Pediatr, 1997. 130(3): p. 378-87.
54. Janik, D.K., et al., A multiplex immunoassay using the Guthrie specimen to detect T-cell deficiencies including severe combined immunodeficiency disease. Clinical chemistry, 2010. 56(9): p. 1460-1465.
55. Collier, F., et al., Flow cytometric assessment of cord blood as an alternative strategy for population-based screening of severe combined immunodeficiency. J Allergy Clin Immunol, 2013. 131(4): p. 1251-2.
56. Buckley, R.H., The long quest for neonatal screening for severe combined immunodeficiency. Journal of Allergy and Clinical Immunology, 2012. 129(3): p. 597-604.
57. Verschuren, M.C., et al., Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. J Immunol, 1997. 158(3): p. 1208-16.

58. Hazenberg, M.D., et al., T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. Journal of Molecular Medicine, 2001. 79(11): p. 631-640.
59. Institute, C.a.L.S., Newborn Bloot Spot Screening for Severe Combined Immunodeficiency by Measurement of T-cell receptor Excision Circles (new version, unpublished). 2021.
60. Douek, D.C., et al., Changes in thymic function with age and during the treatment of HIV infection. Nature, 1998. 396(6712): p. 690-695.
61. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. Journal of Allergy and Clinical Immunology, 2005. 115(2): p. 391-398.
62. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunological Reviews, 2019. 287(1): p. 241-252.
63. Knight, V., et al., Follow-Up for an Abnormal Newborn Screen for Severe Combined Immunodeficiencies (NBS SCID): A Clinical Immunology Society (CIS) Survey of Current Practices. International journal of neonatal screening, 2020. 6(3): p. 52.
64. Chong, H.J., S. Maurer, and J. Heimall, What to Do with an Abnormal Newborn Screen for Severe Combined Immune Deficiency. Immunol Allergy Clin North Am, 2019. 39(4): p. 535-546.
65. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. The Journal of allergy and clinical immunology, 2017. 139(3): p. 733-742.
66. Patrawala, M. and L. Kobrynski, Nonsevere combined immunodeficiency T-cell lymphopenia identified through newborn screening. Curr Opin Allergy Clin Immunol, 2019. 19(6): p. 586-593.
67. Thomas, C., et al., A Severe Neonatal Lymphopenia Associated With Administration of Azathioprine to the Mother in a Context of Crohn's Disease. J Crohns Colitis, 2018. 12(2): p. 258-261.
68. Kuo, C.Y., et al., Profound T-cell lymphopenia associated with prenatal exposure to purine antagonists detected by TREC newborn screening. The Journal of Allergy and Clinical Immunology: In Practice, 2017. 5(1): p. 198-200.
69. Mauracher, A.A., et al., Causes of low neonatal T-cell receptor excision circles: A systematic review. J Allergy Clin Immunol Pract, 2017. 5(5): p. 1457-1460.e22.
70. Dorsey, M. and J. Puck, Newborn Screening for Severe Combined Immunodeficiency in the US: Current Status and Approach to Management. International journal of neonatal screening, 2017. 3(2): p. 15.
71. El-Hattab, A.W., M. Almannai, and V.R. Sutton, Newborn Screening: History, Current Status, and Future Directions. Pediatric Clinics of North America, 2018. 65(2): p. 389-405.
72. Jansen, M.E., et al., Expanded Neonatal Bloodspot Screening Programmes: An Evaluation Framework to Discuss New Conditions With Stakeholders. Frontiers in pediatrics, 2021. 9: p. 635353-635353.
73. Therrell, B.L., et al., Current status of newborn screening worldwide: 2015. Seminars in perinatology, 2015. 39(3): p. 171-187.
74. Jansen, M.E., S.C. Metternick-Jones, and K.J. Lister, International differences in the evaluation of conditions for newborn bloodspot screening: a review of scientific literature and policy documents. European journal of human genetics : EJHG, 2016. 25(1): p. 10-16.
75. Wilson, J.M.G., G. Jungner, and O. World Health, Principles and practice of screening for disease / J. M. G. Wilson, G. Jungner. 1968, World Health Organization: Geneva.

76. Andermann, A., et al., Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years. Bull World Health Organ, 2008. 86(4): p. 317-9.
77. Franková, V., et al., Regulatory landscape of providing information on newborn screening to parents across Europe. European Journal of Human Genetics, 2021. 29(1): p. 67-78.
78. Loeber, J.G., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 1. From blood spot to screening result. Journal of inherited metabolic disease, 2012. 35(4): p. 603-611.
79. Burgard, P., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 2. From screening laboratory results to treatment, follow-up and quality assurance. Journal of inherited metabolic disease, 2012. 35(4): p. 613-625.
80. Recommended Uniform Screening Panel. 2018 February 2019 [cited 2020 8 January]; Recommended Uniform Screening Panel]. Available from: https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html.
81. Wilcken, B. and V. Wiley, Fifty years of newborn screening. Journal of paediatrics and child health, 2015. 51(1): p. 103-107.
82. Kwon, J.M., et al., Consensus guidelines for newborn screening, diagnosis and treatment of infantile Krabbe disease. Orphanet journal of rare diseases, 2018. 13(1): p. 30-30.
83. Orsini, J.J., Newborn screening for Krabbe disease: perceived and current ethical issues. Developmental medicine and child neurology, 2019. 61(12): p. 1354-1354.
84. Fischer, K.E. and W.H. Rogowski, Funding decisions for newborn screening: a comparative review of 22 decision processes in Europe. International journal of environmental research and public health, 2014. 11(5): p. 5403-5430.
85. King, J.R. and L. Hammarström, Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. Journal of clinical immunology, 2018. 38(1): p. 56-66.
86. Routes, J.M., et al., Statewide newborn screening for severe T-cell lymphopenia. Jama, 2009. 302(22): p. 2465-70.
87. Routes, J. and J. Verbsky, Newborn Screening for Severe Combined Immunodeficiency. Curr Allergy Asthma Rep, 2018. 18(6): p. 34.
88. Borte, S., et al., Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood, 2012. 119(11): p. 2552-5.
89. Adams, S.P., et al., Screening of neonatal UK dried blood spots using a duplex TREC screening assay. J Clin Immunol, 2014. 34(3): p. 323-30.
90. Audrain, M., et al., Evaluation of the T-cell receptor excision circle assay performances for severe combined immunodeficiency neonatal screening on Guthrie cards in a French single centre study. Clin Immunol, 2014. 150(2): p. 137-9.
91. de Felipe, B., et al., Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol, 2016. 27(1): p. 70-7.
92. Blom, M., et al., An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. Clin Immunol, 2017. 180: p. 106-110.
93. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
94. Thomas, C., et al., Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clin Immunol, 2019. 202: p. 33-39.
95. Richards, S., et al., Diagnosis and management of severe combined immunodeficiency in Australia and New Zealand. J Paediatr Child Health, 2020. 56(10): p. 1508-1513.

96. Chien, Y.-H., et al., Newborn Screening for Severe Combined Immunodeficiency in Taiwan. International Journal of Neonatal Screening, 2017. 3(3): p. 16.
97. Rechavi, E., et al., First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol, 2017. 8: p. 1448.
98. Strand, J., et al., Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol, 2020. 11: p. 1417.
99. Trück, J., et al., Swiss newborn screening for severe T and B cell deficiency with a combined TREC/KREC assay - management recommendations. Swiss Med Wkly, 2020. 150: p. w20254.
100. Health Council of the Netherlands, Neonatal screening: new recommendations. 2015, Health Council of the Netherlands: The Hague.

CHAPTER 2

An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program

Maartje Blom, Ingrid Pico-Knijnenburg, Marja Sijne-van Veen, Anita Boelen, Robbert G.M. Bredius, Mirjam van der Burg and Peter C.J.I. Schielen

Clinical Immunology, 2017; 180: 106-110

ABSTRACT

Newborn screening of severe combined immunodeficiency through the detection of T-cell receptor excision circles will provide the opportunity of treating before the occurrence of life-threatening infections. With the EnLite Neonatal TREC assay (PerkinElmer) and end-point PCR, 39 samples (3.0%) of 1295 heel prick cards of the Dutch newborn screening program required a retest after initial analysis. After retest, 21 samples (1.62%) gave TREC levels below cut-off. A significant reduction in TREC levels was observed in heel prick cards stored for three months (N = 33) and one year (N = 33). Preterm newborns (N = 155) showed significantly lower TREC levels and a higher retest-rate than full-term newborns. Peripheral blood spots of 22 confirmed SCID patients and 17 primary immunodeficiency patients showed undetectable or low TREC levels. These findings suggest that the EnLite Neonatal TREC assay is a suitable method for SCID screening in the Netherlands, thereby providing guidance in the decisions concerning implementation into the Dutch program.

INTRODUCTION

Severe combined immunodeficiency (SCID) comprises a group of heterogeneous genetic disorders of the immune system, characterized by the dysfunction of T-lymphocyte maturation and development. In addition to a lack of T-cell-mediated immunity, SCID patients can present with various subtypes of disrupted differentiation or function of B-lymphocytes and natural killer cells [1]. Newborns with SCID usually present with severe infections and failure to thrive during the first months of life. Affected children face a fatal outcome unless their immune system is replaced by hematopoietic stem cell transplantation (HSCT) or gene therapy [2]. Transplantation outcome improves significantly if children receive a transplant before the age of 3.5 months and prior to the occurrence of the first infection [3]. Moreover, it was shown that SCID babies diagnosed at birth due to positive family history have significantly improved overall survival and transplantation outcome compared to the firstborn affected family member [4]. Screening of SCID leading to early diagnosis has shown to be cost-effective in spite of a low incidence of the disease [5]. These findings imply that SCID is a suitable candidate for newborn screening.

Worldwide, many SCID screening pilots have been conducted and implementation of SCID in the newborn screening programs is discussed extensively [6-11]. In 2010, SCID was added to the Recommend Uniform Newborn Screening Panel of the United States resulting in the incorporation of SCID in the national screening programs of > 33 states [6]. A recent review of the Dutch Health Council also identified SCID as a suitable candidate for newborn screening. Consequently, in July 2015, the Dutch Ministry of Health adopted the advice of the Dutch Health Council to incorporate SCID in the Dutch newborn screening program.

Newborn SCID screening is based on the detection of T-cell receptor excision circles (TRECs) in dried blood spots using polymerase-chain reaction (PCR) techniques. TRECs are stable circular DNA fragments formed during the T-cell receptor rearrangement process, thereby serving as a biomarker for newly formed T-lymphocytes. Healthy newborns present with TRECs in large quantities, while SCID patients show low or undetectable TREC levels [12]. A TREC assay for newborn screening is now commercially available in the form of the EnLite™ Neonatal TREC kit (PerkinElmer, Turku, Finland). Confirmatory testing such as flow cytometry and gene sequencing should be performed after initial TREC screening to confirm the diagnosis of SCID and exclude other T-lymphocytopenia associated disorders.

In anticipation of the implementation of SCID screening into the Dutch newborn screening program, this study aims to obtain more experience with the available TREC assay. We demonstrated that the EnLite Neonatal TREC assay is a suitable method for newborn screening for SCID in the Netherlands, with applicability of the screening protocol for the Dutch screening laboratories as an important finding of this study.

METHODS

Study population

Anonymized (clinical follow-up of putative positive results was not an aim of this part of the study) fresh heel prick cards (N = 1295) from the Dutch Newborn Screening program of the regions Gelderland and Utrecht were used. Dried blood spots were collected between 72 and 168 hours after birth and analyzed in singlicate within five days after collection. All parents or representatives gave informed consent for the use of patient material for scientific research. The use of anonymized heel prick cards was approved by the Working group Scientific Research Newborn Screening of the Dutch screening organization. Heel prick cards from newborns who received a blood transfusion were excluded from the study. Secondly, singular analysis of heel prick cards stored for two weeks (N = 61), one month (N = 63), three months (N = 33) and one year (N = 33) at 4˚C was carried out, to evaluate the effects on samples that are in transport for prolonged periods of time or lifted from long term storage for e.g. confirmatory re-analysis of newborn samples or retrospective studies. Heel prick cards of 155 preterm newborns (birthweight ≤ 2500 g and gestational age ≤ 36.0 weeks) stored at 4 ˚C and not older than two months were included. Filter paper cards (PerkinElmer 226 paper, PerkinElmer, Shelton, USA) were spotted with peripheral blood from 22 patients with a clinical, genetically confirmed, SCID diagnosis, (affected genes: ADA N = 2, RAG1 N = 6, RAG2 N = 2, IL2Rg N = 4, JAK3 N = 2, XLF N = 2, Artemis N = 2, CD3E N = 2) and of 27 patients with a primary immunodeficiency (PID), potentially SCID, however not confirmed by genetic analysis. These cards were included in the analysis. Samples were obtained according to the rules of the Medical Ethical Committee of the Erasmus MC, Rotterdam. At the Erasmus MC, blood or bone marrow samples of these 49 patients clinically suspected of a potential immunodeficiency, were analyzed by flow cytometry. Based on the flow cytometric results, certain genes were selected and studied by PCR or Sanger sequencing techniques resulting in the SCID diagnosis above.

Reference samples were kindly provided by the Newborn Screening Translational Research Initiative at the Center for Disease Control and Prevention (CDC, Atlanta, Georgia) as part of the Model Performance Evaluation Survey (MPES, the MPES is an

international collaborative research project among newborn screening laboratories). The set consisted of nine TREC reference dried blood spots. Six specimens were created out of cord blood: two specimens with TREC levels close to the cut-off (S356 and L4), two specimens with medium TREC levels (Hi and S339), one specimen with TREC levels below average (L2) and one SCID-like specimen with low or no TREC levels (SCID 2). Two samples were created out of peripheral blood mononuclear cells (PBMC) depleted blood to which a known number of TREC copies was added (B-TREC Cal 3250 TREC copies/µl blood and B-TREC Cal 5 62.5 TREC copies/µl of blood). Lastly, one blood specimen (blood with buffy coat removed, named UnSat) with TREC and reference gene levels below the cut-off levels was included.

Calibration dried blood spot (DBS) samples (PerkinElmer) with TREC levels of 28 copies/µl (A), 167 copies/µl (B) and 578 copies/µl (C) were included in triplicate. Control spots (PerkinElmer) C1 (low TREC, low β-actin), C2 (no TREC, normal β-actin) and C3 (normal TREC, normal β-actin) were included in duplicate. Both calibration spots and control spots were prepared from porcine whole blood with a hematocrit level of 48–55% containing purified salmon-sperm, TREC and β-actin DNA. Cards with patient material were stored at room temperature according to standards of the Erasmus MC, while the original heel prick cards and CDC reference materials were stored at 4–7 ˚C in accordance with the screening laboratory procedures. Calibration- and control spots were stored at −30 ˚C to −16 ˚C as indicated in the kit instructions.

DNA elution from dried blood spot punches

The TREC assay was performed according to the EnLite Neonatal TREC kit instructions (Perkin Elmer). From each heel prick card single 1.5 mm discs were directly punched in a 96 wells plate (3410–0010, Bio-Rad, Veenendaal, the Netherlands) using a Wallac DBS puncher (1296-071, PerkinElmer). To prevent any static interference, plates were passed through an ionizing gate (Eltex Elektrostatik GmbH, Weil am Rhein, Germany). Blank reactions without sample material were carried out in triplicate to check for contamination. Elution buffer and reagent mixture were prepared according to the kit instructions (PerkinElmer) in a pre-PCR area. After punching, 10 µl of Elution buffer (ready for-use buffer with MgCl2) was added to each well of the PCR-plate. PCR-plates were sealed, centrifuged (500 × g, 60 s) and incubated for 45 min at 98 ˚C and 2 min at 4 ˚C in a Bio-Rad Thermal Cycler S1000 (Bio-Rad, Veenendaal, the Netherlands).

PCR amplification and signal measurement

After elution, the seal was removed and 20 µl of reagent mixture was added to each well of the PCR-plate. Next, the plate was resealed, centrifuged briefly (1 min, 500g) and placed in the Thermal Cycler. Amplification reactions consisted of an initial

denaturation-cycle of 5 min at 98 ˚C and 37 cycles of 15 s at 98 ˚C, 1 min at 62.5 ˚C and 15 s at 72 ˚C. Following the amplification step, probe hybridization was allowed for 5 min at 95 ˚C, 60 min at 35 ˚C and 5 min at 23 ˚C. After completion of amplification and hybridization, the PCR plate was centrifuged for 2 min at 500g and was placed in the Victor EnLite fluorometer (model 1420-0220, PerkinElmer) to measure the fluorescence signal of TREC and β-actin. Calibration curves were created by the EnLite Workstation software, based on fluorescence counts measured at 615 nm, 665 nm, and 780 nm. The intensity of the fluorescence signal is directly related to the number of TREC/β-actin DNA copies/µl. For the corrected results, the TREC and β-actin levels of the samples were fitted against the ArcSinh transformed concentrations of the calibration spots by using unweighted linear regression. The control dried blood spots were required to have the correct number of TREC- and β-actin copies/µl as a quality control. The measurement of β-actin signal is only relevant if TREC levels of a sample are below cut-off in the initial analysis. The analysis should be repeated in duplicate from the same heel prick card if the β-actin signal is too low. Results were considered invalid or inconclusive in case one of the duplicate spots gave β-actin levels below a cut-off (40 copies/µl). After this retest in duplicate, the β-actin levels are interpreted to verify whether elution and amplification were sufficient. In this study, an experimental TREC cut-off level of 40 copies/µl was used to distinguish screen positive samples, based on advisory information from the kit insert of the manufacturer. The manufacturer advices to perform a large sample size pilot study to establish the preferred cut-off value based on the normal population distribution in order to establish a reasonable referral rate without any loss of cases.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS version 22.0 (SPSS Inc., Chicago, Illinois). Two-tailed statistical analysis was performed and $P < 0.05$ was considered statistically significant. The Mann-Whitney test and (un)paired t-test were used to determine the difference in TREC levels between fresh heel prick cards and stored heel prick cards and heel prick cards of preterm newborns and full-term newborns. Intra- and inter-assay variation was determined using logarithmically transformed data. All transplantations were performed according to European society for Blood and Marrow Transplantation guidelines. Blood samples were routinely obtained and analyzed after approval by the institutional review board (protocol P01.028). Informed consent was provided by the patient and/or a parent or guardian.

RESULTS

Calibration curves were generated using the TREC- and β-actin levels of the blank reactions and calibration dried blood spots A–C. The correlation coefficients of the calibration curve of 28 runs were typically above 0.9900 for TREC (range 0.9760–0.999) and 0.9880 for β-actin (range 0.9679–0.9999).

To determine the precision of the TREC assay, assay variation was estimated using the control samples (C1, C2, C3) included in each run. The control samples C1, C2 and C3 gave results in accordance with the pre-set targets with an intra-assay variation of the C1 control of 0.46 Ln copies/µl and an intra-assay variation of the C3 control of 0.45 Ln copies/µl. Average based inter-assay variation of the C1 control was 0.23 Ln copies/µl and 0.18 Ln copies/µl for the C3 control. The TREC median of the C2 control was 1.0 copy/µl while 94.6% of all measured values presented ≤ 5 copies/µl.

The mean TREC level of 1295 anonymized Dutch heel prick cards was 111.8 copies/µl blood (median TREC: 96 copies/µl). With the TREC cut-off set at 40 copies/µl blood, 39 samples (3.0%) required a retest after the initial analysis (Figure 1). After retest, 21 samples (1.62%) gave TREC levels in either duplicate spot below cut-off and β-actin levels in both duplicates above the cut-off of 40 copies/µl. Only one sample of the 1295 dried blood spots gave β-actin below cut-off and would therefore require a second heel prick.

The TREC cut-off level based on the 2.5 percentile of the data analyzed with the EnLite Neonatal TREC assay was 39 copies/µl. Table 1 shows the decrease in total numbers of annual referrals when different TREC cut-off levels are applied.

To determine the effect of storage time on TREC levels in dried blood spots, a comparison between fresh and stored heel prick cards was performed. In a first experiment the median TREC levels of fresh heel prick cards was 84 copies/µl blood. After storage for two weeks it was 90 copies/µl blood. Moreover, the median TREC level of the fresh heel prick cards was 85 copies/µl with a median TREC level after storage for one month of 87 copies/µl. No significant difference in TREC levels was observed between fresh heel prick cards and heel prick cards stored for two weeks ($P = 0.86$) or one month ($P = 0.10$).

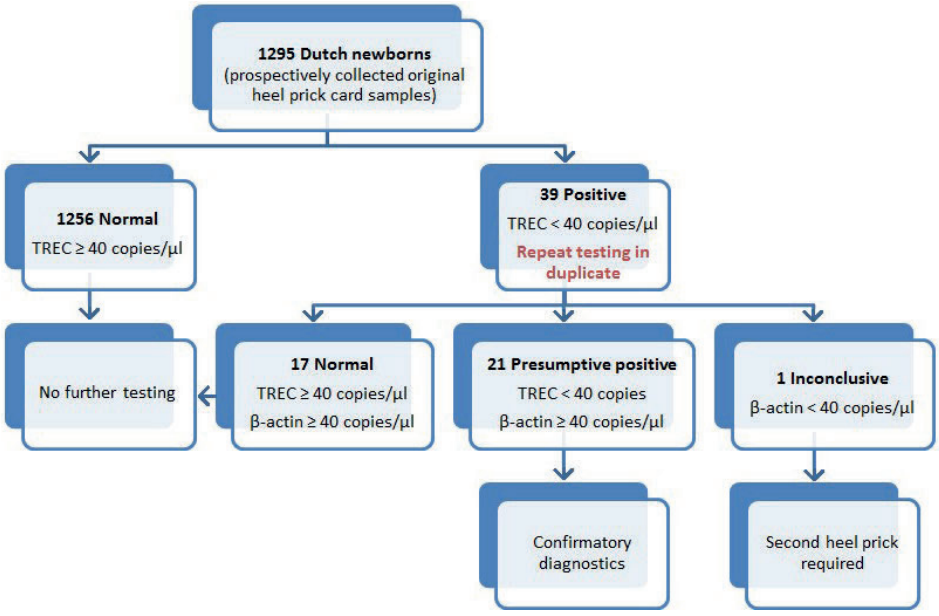

Figure 1. Flow chart of the EnLite Neonatal TREC assay, including results of 1295 fresh anonymized heel prick cards from Dutch newborns.

Table 1. Percentage of positives after initial testing, percentage of presumptive positives after second round testing and the total number of annual referrals with different TREC cut-off levels based on 175,181 births in 2014 [14].

	40 copies/µl	39 copies/µl	35 copies/µl	30 copies/µl	25 copies/µl	22 copies/µl
Percentage of positives after the initial test (%)	3.0	2.5	1.54	0.77	0.39	0.15
Percentage of presumptive positives/ referral rate (%)	1.62	1.31	0.69	0.54	0.23	0.08
Annual referrals (N)	2838	2295	1209	946	403	140

A second comparison was made between fresh heel prick cards and unpaired heel prick cards stored for 3 months and one year at 4–7 ˚C, respectively. Data in Figure 2 show a decrease in TREC levels of heel prick cards stored for 3 months and one year. The median TREC level of the fresh heel prick cards (N = 90) that were included in the same runs was 109.5 copies/µl. The median TREC level of the heel prick cards stored for three months (N = 33) was 69 copies/µl (P = 0.0008), and of cards stored for one year (N = 33) was 69 copies/µl as well (P < 0.0001).

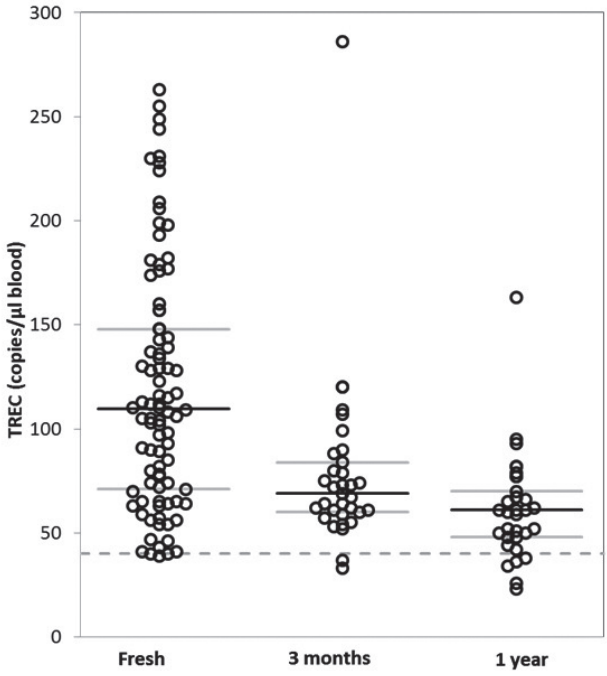

Figure 2. Results of the analysis of 90 fresh heel prick cards using the EnLite Neonatal TREC assay; 33 heel prick cards stored for three months and 33 heel prick cards stored for one year. Horizontal black lines show the median TREC levels, horizontal grey lines depict the standard deviation.

Samples of all 22 genetically confirmed SCID patients (with T lymphocytopenia and known underlying mutations) had low or absent TREC levels and far below the cut-off level (range 0–4 TREC copies/µl) (Figure 3). In addition, a series of 27 samples of PID patients without a defined genetic diagnosis were tested. In 14 patients, the TREC level was below 7 copies/µl. Twelve of these patients were clinically suspected for SCID and had strongly reduced T-cell numbers. Initial genetic testing did not reveal the identification of the genetic defect, but further genetic testing via whole exome sequencing is now considered. Two of the 14 samples with TREC < 7 copies/µl were from adult patients with common variable immunodeficiency (CVID). It is known that a part of CVID patients have reduced TREC and KREC levels [13]. The remaining 13 PID patients had TREC levels above 25.5 copies/µl; none them had T-cell lymphopenia. The clinical diagnosis varied between CVID (N = 5), hyper IgM syndrome (N = 2), suspicion for SCID (which was not confirmed after flowcytometric phenotyping) (N = 2), and unclassified PIDs (N = 4). The mean TREC level of confirmed SCID patients was 0.34 copies/µl blood (median: 0 copies/µl). The mean TREC level of PID patients with inconclusive diagnoses was 46 copies/µl blood (median: 6.3 copies/µl).

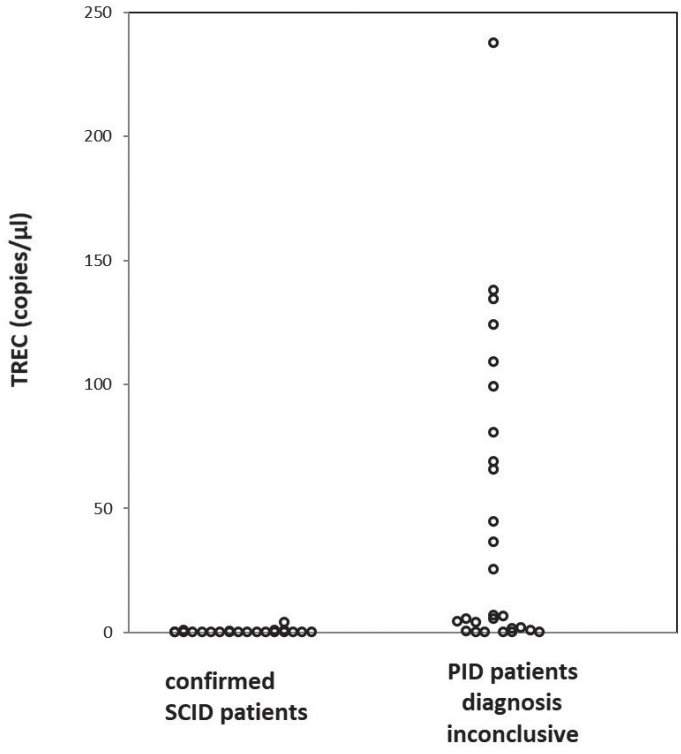

Figure 3. Results of the analysis of peripheral blood samples of 22 genetically confirmed SCID patients and 27 PID patients without genetic diagnosis using the EnLite Neonatal TREC assay.

Table 2 shows data of TREC analyses of samples of preterm newborns. The mean TREC level of 155 heel prick cards of preterm newborns was 64.9 copies/µl with a median of 55 copies/µl, compared to (112 copies/µl blood in full-term infants (median: 96 copies/µl, $P < 0.0001$)). Of the 155 samples, 45 specimens gave TREC levels below the cut-off level of 40 copies/µl resulting in a retest-rate of 29%. A 9.6 fold difference was observed compared to the retest-rate of full-term infants (3.0%).

The nine TREC reference DBS samples were analyzed in two different runs in duplicate. Eight out of nine specimens gave results within the preset categories. Four cord blood specimens showed normal results (TREC/β-actin levels ≥ cut-off) with medium TREC levels, TREC levels close to cut-off and TREC levels below average, respectively (L4, Hi, S339, L2). The SCID-like specimen (SCID2) gave a presumptive positive result with TREC levels close to zero and β-actin levels within the standard reference range. The PBMC-depleted blood samples gave TREC levels according to the added number of cells (B-TREC Cal 3250 TREC cells/µl and B-TREC Cal 5 62.5 TREC cells/µl). The blood

sample with the buffy coat removed (UnSat) gave TREC and β-actin levels below the cut-off levels (as expected). The result of only one cord blood specimen (S356) differed from the target values in two runs in duplicate. While this sample should have given TREC levels close to cut-off (40 copies/µl), the results showed TREC levels far above cut-off with a mean TREC level of 270 copies/µl.

Table 2. Descriptive statistics for TREC levels (copies/µl) of 155 preterm samples, categorized by gestational age.

Gestational age	Number of samples	Mean (TREC copies/µl)	Median (TREC copies/µl)
≤28 weeks	16	44.5	45.5
29–32 weeks	61	60.1	50.0
33–36 weeks	78	72.8	59.0

DISCUSSION

In this study, an evaluation of a TREC SCID screening assay was performed evaluating the applicability in the Dutch screening program. Of the 39 fresh heel prick specimens that gave TREC levels below the pre-set cut-off of 40 copies/µl blood, 21 samples presented presumptively positive, 17 samples gave normal results and one sample presented as inconclusive after re-analysis in duplicate, resulting in a referral-rate of 1.62%. In routine screening, these presumptively positive newborns would be referred for confirmatory diagnostics such as flow cytometry or gene sequencing. With 175,181 births in 2014 [14], a referral rate of 1.62% would result in annually about 2838 referrals for follow-up diagnostics, or 55 infants per week distributed among the five Dutch screening laboratories.

Results of experiments on the influence of storage times on TREC levels confirmed previous unpublished results by the manufacturer that indicate that TREC levels decrease with increasing storage time, but not for storage times up to one month at room temperature. As the Dutch newborn screening program receives heel prick cards of the Caribbean Netherlands, with shipping times up to two weeks, we thus expect that these samples will not yield compromised TREC levels due to prolonged storage in transportation.

The cut-off level based on the 2.5 percentile of the data analyzed with the EnLite Neonatal TREC assay was 39 copies/µl. With this new cut-off level the retest-rate would drop to 2.5%. Table 1 shows the decrease in total numbers of annual referrals when different TREC cut-off levels are applied. A high referral rate could result in an excessive workload

for downstream referral centers. Therefore, lowering the TREC cut-off level is worth consideration. Since SCID patients present in most cases with very low or undetectable TREC levels, lowering the TREC cut-off would most likely not result in an increase of false-negative results. At a TREC cut-off level of 22 copies/µl the number of presumptive positives/referral rate would be 0.08%, which would result in an annual 143 referrals for follow-up diagnostics. These numbers are comparable with the referral rates of pilot studies in the UK (0.04%), California (0.02%) and Wisconsin (0.03%) [10]. The exact number of presumptively positive samples and the exact referral rate can only be established once a pilot is carried out in the Netherlands with a considerably larger sample size (e.g. 15–30.000). The inconclusive sample showed β-actin levels below cut-off without agreement between duplicates in three analyses. In the regular screening program, a second heel prick should be requested if samples present inconclusive after being analyzed in duplicate. With approximately 178,000 samples being analyzed annually, this would result in 137 second heel pricks each year. If the second heel prick shows inconclusive results as well, flow cytometry should be performed.

Consistent with previous studies [15-17], NBS samples of Dutch preterm newborns had low TREC levels and a higher retest-rate than samples of newborns with a full-term pregnancy. There are several options in which the SCID screening algorithm could be adjusted based on these findings. A first possibility would be to request a second heel prick of all preterm newborns that present presumptively positive after the second analysis. This second heel prick could be taken immediately or after the preterm newborn has reached the adjusted gestational age of 37 weeks (in accordance with the screening algorithm of the state of Delaware [6]). Previous research showed an increase and normalization of the concentration of T-cells as the age of the preterm newborn advanced [18]. In other states, such as Connecticut and New York, the cut-off level for preterm newborns was lowered in order to reduce the retest-rate [6]. Based on our data, the 2.5 percentile-based cut-off level for preterm newborns would be < 16 copies/µl. Finally, the same screening algorithm and cut-off levels for both full-term as well as preterm samples could be used (comparable to the algorithm used in the state of Michigan). In Michigan, a cut-off level was chosen at which newborns with a birth weight ≤ 2500 g showed a five-fold higher rate of false positive screening results compared to newborns with a birth weight > 2500 g. To prevent delayed diagnosis of preterm newborns with SCID, this balance between the number of false-positive and false-negative results was deemed acceptable [19].

In all 22 samples of confirmed SCID patients, the EnLite Neonatal TREC assay showed absent TREC levels or levels far below cut-off, indicating the applicability of the assay for detection of newborns with SCID. Of the 27 PID patients without a genetic diagnosis, 14 samples gave TREC levels below the cut-off level of 7 copies/µl. Twelve of these patients were clinically suspected for SCID and had strongly reduced T-cell numbers, however, Initial genetic testing did not reveal the identification of a genetic defect. Further genetic testing via whole exome sequencing is now considered. The other two samples were from CIVD patients with reduced T-cell numbers [13]. Previous studies suggested that TREC analysis of dried blood spots would be unable to detected newborns with an adenosine deaminase deficient (ADA) type SCID [20]. In this study, two patients with a mutation in the ADA gene (c.956_960delAAGAG and c.302G > A) were both detected with EnLite Neonatal TREC assay, confirming that although delayed-onset ADA deficiency might not be detected by TREC quantification, ADA-deficient newborns with a T-cell deficient phenotype will be identified using a TREC assay.

In conclusion, the first results with TREC assay imply that EnLite Neonatal TREC assay is a suitable method for newborn screening for SCID in the Netherlands. The introduction of SCID in the Dutch screening program is already sanctioned by the Dutch minister of Public Health, Welfare, and Sports [21]. With the findings of the current study, the first advisory information concerning the TREC assay for SCID screening is provided. With this knowledge, a first step is made in the integration of SCID screening in the Dutch screening program.

REFERENCES

1. B.J. Buelow, J.M. Routes, J.W. Verbsky, Newborn screening for SCID: where are we now? Expert. Rev. Clin. Immunol, 2014. 12:p. 1649-57.
2. R. Somech, et al., Newborn screening for severe T and B cell immunodeficiency in Israel: a pilot study, Isr. Med. Assoc. J, 2013. 15: p. 404–409.
3. S.-Y. Pai, et al., Transplantation outcomes for severe combined immunodeficiency, 2000–2009, N. Engl. J. Med, 2014. 371: p. 434–446.
4. L. Brown, et al., Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening, Blood, 2011. 217: p. 3243–3246.
5. S.A. McGhee, E.R. Stiehm, E.R.B. McCabe, Potential costs and benefits of newborn screening for severe combined immunodeficiency, J. Pediatr, 2005. 147: p. 603–608.
6. A. Kwan, et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States, JAMA, 2014. 312: p. 729–738.
7. M. Audrain, et al., Evaluation of the T-cell receptor excision circle assay perfor- mances for severe combined immunodeficiency neonatal screening on Guthrie cards in a French single centre study, Clin. Immunol, 2014. 150 p. 137–139.
8. Y.-H. Chien, et al., Incidence of severe combined immunodeficiency through new- born screening in a Chinese population, J. Formos. Med. Assoc, 2015. 114: p. 12–16.
9. P. Olbrich, et al., A first pilot study on the neonatal screening of primary immunode- ficiencies in Spain: TRECS and KRECS identify severe T- and B-cell lymphopaenia, An. Pediatría (English Ed.), 2014. 81: p. 310–317.
10. S.P. Adams, et al., Screening of neonatal UK dried blood spots using a duplex TREC screening assay, J. Clin. Immunol, 2014. 34: p. 323–330.
11. A. Kwan, et al., Successful newborn screening for SCID in the Navajo Nation, Clin. Immunol, 2015. 158: p. 29–34.
12. H.B. Gaspar, L. Hammarström, N. Mahlaoui, M. Borte, S. Borte, The case for manda- tory newborn screening for severe combined immunodeficiency (SCID), J. Clin. Immunol, 2014. 34: p. 393–397.
13. F. Serana, et al., Thymic and bone marrow output in patients with common variable immunodeficiency, J. Clin. Immunol, 2011. 31: p 540–549.
14. Centraal Bureau voor de Statistiek, CBS StatLine - Geboorte; kerncijfers at http://statline.cbs.nl/StatWeb/publication/?VW=T&DM=SLNL&PA=37422ned&D1=0,4-5,7,9,11,13,17,26,35,40-41&D2=0,10,20,30,40,(l-4)-l&HD=090218-0953&HDR= G1&STB=T 2016.
15. B.H. Vogel, et al., Newborn screening for SCID in New York state: experience from the first two years, J. Clin. Immunol, 2014. p. 289–303.
16. J.W. Verbsky, et al., Newborn screening for severe combined immunodeficiency; the Wisconsin experience (2008–2011), J. Clin. Immunol, 2012. 32 p. 82–88.
17. J.M. Routes, et al., Statewide newborn screening for severe T-cell lymphopenia, JAMA, 2009. 302: p. 2465–2470.
18. A. Kwan, et al., Newborn screening for severe combined immunodeficiency and T- cell lymphopenia in California: results of the first 2 years, J. Allergy Clin. Immunol, 2013. 132: p. 140–150.e7.

19. U. Duffner, Optimizing newborn screening for severe combined immunodeficiency - the Michigan experience, Pediatrics, 2013. 131: p. 1298.
20. G. la Marca, et al., Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency, J. Allergy Clin. Immunol, 2013. 135: p. 1604–1610.
21. Letter of the Dutch Minister of Health (mw. drs. E.I. Schippers) to the Chairman of the House of Representatives of the Netherlands on the expansion of the newborn screening program, 9th of July at http://artsenjgz.nl/nieuwsbericht/reactie-minis- ter-van-vws-op-advies-gezondheidsraad-over-14-nieuwe-aandoeningen-in-de- hielprikscreening/N. 2015

CHAPTER 3

Introducing newborn screening for severe combined immunodeficiency (SCID) in the Dutch neonatal screening program

Maartje Blom, Robbert G.M. Bredius, Gert Weijman, Eugènie H.B.M. Dekkers, Evelien A. Kemper, M. Elske van den Akker-van Marle, C.P.B. (Kitty) van der Ploeg, Mirjam van der Burg and Peter C.J.I. Schielen

International Journal of Neonatal Screening, 2018; 4(4): 40

ABSTRACT

The implementation of newborn screening for severe combined immunodeficiency (SCID) in the Netherlands is a multifaceted process in which several parties are involved. The Dutch Ministry of Health adopted the advice of the Dutch Health Council to include SCID in the Dutch newborn screening program in 2015. As newborn screening for SCID is executed with a new, relatively expensive assay for the Dutch screening laboratory, an implementation pilot study is deemed instrumental for successful implementation. A feasibility study was performed in which the practicalities and preconditions of expanding the newborn screening program were defined. Cost-effectiveness analysis (CEA) indicated that SCID screening in the Netherlands might be cost-effective, recognizing that there are still many uncertainties in the variables underlying the CEA. Data and experience of the pilot study should provide better estimates of these parameters, thus enabling the actualization of CEA results. Prior to the implementation pilot study, a comparison study of two commercially available SCID screening assays was performed. A prospective implementation pilot study or so-called SONNET study (SCID screening research in the Netherlands with TRECs) started in April 2018 and allows the screening for SCID of all newborns in three provinces of the Netherlands for one year. Based on the results of the SONNET study, the Dutch Ministry of Health will make a final decision about national implementation of newborn screening for SCID in the Netherlands.

INTRODUCTION

This article provides a brief overview of the developments in the Netherlands with regard to newborn screening for severe combined immunodeficiency (SCID) for the special themed "Newborn Screening for primary immunodeficiency diseases—Past, Present and Future" issue. The Dutch newborn screening program started in 1974 with screening for phenylketonuria (PKU). Since then, the number of disorders in the newborn screening program expanded significantly and newborns in the Netherlands are now being screened for nineteen disorders. Each year approximately 175,000 newborn blood spot screening tests are performed. Participation in the newborn screening program has remained stable over time, and was approximately 99.2% in 2016 [1]. Newborn blood spot collection is carried out as soon as possible within 72 to 168 hours after birth. Newborn screening analyses are performed in one of the five screening laboratories in the Netherlands. The screening laboratory of the National Institute of Public Health and the Environment (RIVM) serves as a reference laboratory. The primary process of newborn screening in the Netherlands is depicted in Figure 1. At the national level, the screening program is organized by the RIVM's Centre for Population Screening (CvB) on behalf of the Dutch Ministry of Health, Welfare and Sport. The Programme Committee for Newborn Blood Spot Screening, which was established by the RIVM CvB, advises the RIVM with regard to the program's national coordination. Neonatal screening is dynamic and subject to change. Treatment options and screening test methods for certain disorders have improved significantly over the past years [2, 3]. The development of a detection method for SCID [4, 5] and the implementation of this test method in the newborn screening programs of the United States [6] and other countries [7, 8] raised public and expert attention to study the implementation of newborn screening for SCID in the Dutch newborn screening program.

Newborn Screening Recommendations by the Health Council of the Netherlands

The Health Council of the Netherlands, established in 1902, is an independent scientific advisory body whose remit it is to advise the government and Parliament with respect to public health issues and health (care) research. Previous reports of the Health Council in 2005 and 2010 resulted in the expansion of the newborn screening program to seventeen disorders [9, 10]. In 2012, the Minister for Health, Welfare and Sport asked the Council for a new advisory report on newborn screening that would mainly focus on the recommendation of new disorders to be implemented in the newborn screening program. Other issues that also were requested to be addressed were the criteria for inclusion of disorders in neonatal screening, conditions currently eligible for inclusion in screening, and how incidental secondary findings should be dealt with in

the program. The report 'Neonatal screening: new recommendations' came out in 2015, in which the Committee recommended to add fourteen new conditions to the neonatal screening program. These new conditions are alpha- and beta-thalassemia, carnitine acylcarnitine translocase deficiency (CACT), carnitine palmitoyltransferase deficiency type 1 (CPT1), carnitine palmitoyltransferase deficiency type 2 (CPT2), galactokinase deficiency (GALK), guanidinoacetate methyltransferase deficiency (GAMT), beta-ketothiolase deficiency (BKT), methylmalonic acidemia (MMA), mucopolysaccharidosis type 1 (MPS I), organic cation transporter 2 deficiency (OCTN 2), propionic acidemia (PA), X-linked adrenoleukodystrophy (X-ALD) and SCID [11]. The report also included advice about X-linked a-gammaglobulinemia (XLA), stating that a research study of the test characteristics of the kappa-deleting recombination excision circles (KREC) test should be initiated before inclusion of XLA in the neonatal screening program can be reconsidered. The Committee stated that newborn screening for SCID would prevent significant, irreversible damage and yield substantial health gains for the affected child. Although the detection of T-cell receptor excision circles (TRECs) by PCR is more complicated and expensive than other neonatal test methods, it would seem to stay within acceptable limits of efficacy. The Committee did recommend a more extensive pilot study and an exact cost-benefit analysis as part of the implementation process.

The Response of the Dutch Ministry of Health, Welfare and Sport

On 9 July 2015, the Dutch Ministry of Health, Welfare and Sport published a policy position paper entitled "Newborn blood spot screening". In this briefing, the Minister adopted the advice of the Health Council to extend the newborn blood spot screening with fourteen new disorders. The Minister subdivided the implementation process of these disorders into three phases, involving short, medium and long preparation times. Although other countries have already screened for SCID for many years [6], TREC detection based on PCR is a relatively expensive test method, which has yet to be validated in the Dutch screening laboratory. A thorough process of implementation of newborn screening for SCID would therefore require a long preparation time. The Minister did, however, urge to give priority to the implementation of SCID screening, as SCID would be the first disorder in the newborn screening program that could not only be treated but completely cured. The Minister also asked the Centre for Population Screening to carry out a feasibility study to determine the practicalities involved with the implementation of fourteen new disorders [12].

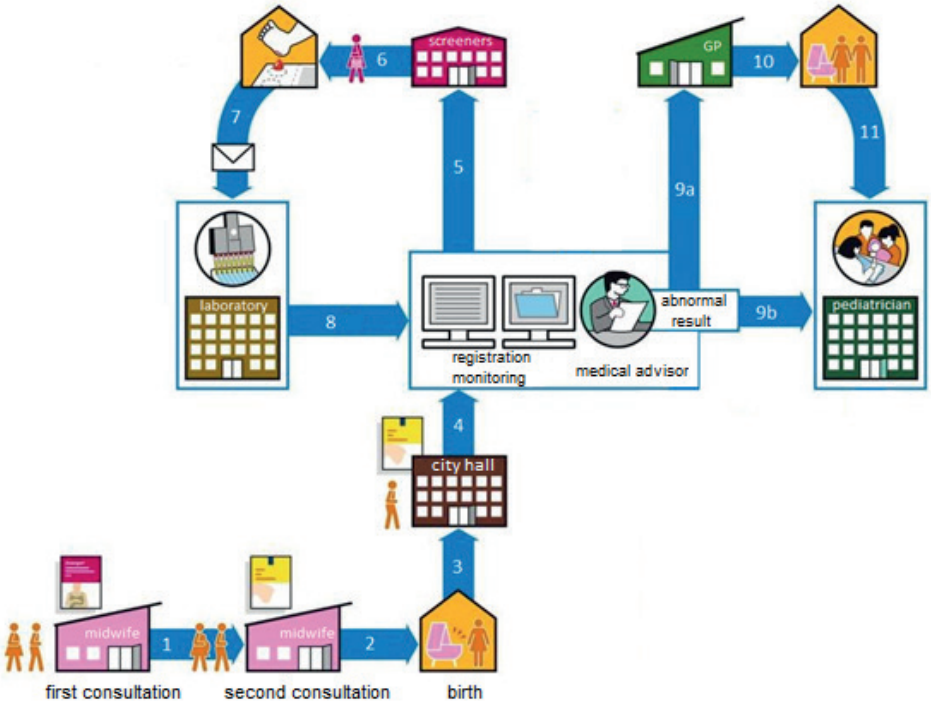

Figure 1. The primary process of the Dutch newborn screening program. Expecting parents will receive information about the newborn screening program during the first (1) and second consultation with the midwife (2). The first information brochure briefly mentions newborn screening, whereas the second brochure elaborates on the objectives, disorders and newborn screening process. During registration of a newborn at city hall, the second information brochure will be handed out as well (3). The screening organizations (5) will be informed about the registration of the newborn at city hall (4), after which screeners will visit the parents at home or in the hospital (6). They will perform the heel prick and send the heel prick card by post to one of the five screening laboratories (7). The heel prick cards are then analyzed and the results are registered in the laboratory information system (LIMS) and the national monitoring database Praeventis (8). Abnormal results are forwarded to the general practitioner (GP) (9a) and pediatrician (9b) by the medical advisor. Medical advisors coordinate logistics of the referral procedure. GPs will visit the parents and their newborn (10) and inform them about the referral of their newborn to the pediatrician within the pre-set referral time (11).

Feasibility Study by the Centre for Population Screening

The Centre for Population Screening of the RIVM carried out a feasibility study commissioned by the Ministry of Health to investigate the practicalities and preconditions of expanding the newborn screening program. The expansion is a complex process due to the large number of disorders, changes in logistics and organization of screening laboratories, availability and quality of test methods and new follow-up procedures. The feasibility study report was published in July 2017 and stated that implementation of the fourteen new disorders would only be feasible in a phased manner and if the following conditions are met: adequate staffing levels and financial means, the availability of flexible IT amenities, and a good interface with the health services [13]. The report emphasized the importance of second tier or even third tier testing and post-analytic tools to prevent large numbers of false positive referrals. It is of great importance that the present neonatal screening program is not affected by the planned expansion and its associated preparations. At the end of each preparatory phase, the Secretary of State for Health, Welfare and Sport must decide whether the condition can enter the implementation phase or whether further research is required. Alpha- and beta-thalassemia were already implemented in the newborn screening program in the beginning of 2017. The proposed planning of implementing the remaining disorders follows a five-year plan. Implementation of CPT1, MMA and PA is deemed feasible by the end of 2019. Other disorders will follow by the end of 2020 (MPS I and GALK), by the end of 2021 (CACT, CPT, BKT, OCTN2, SCID and X-ALD) and finally by the end of 2022 (GAMT). The total costs of expanding the newborn screening program are estimated at 14 million euros over the five-year period. The Centre for Population Screening dedicated a separate chapter to the implementation of SCID screening, as the pilot study for SCID screening requires new equipment, adjustments in the screening laboratory, training of staff, changes in the laboratory information system (LIMS) and the monitoring database Praeventis, and new referral and follow-up protocols. Parents should be informed about the SCID screening pilot during their pregnancy and after birth. This means that new brochures and leaflets with comprehensible information for parents had to be developed that fitted in to the existing information framework of the newborn screening program. Information material for professionals and health care providers about SCID and the SCID screening pilot had to be developed as well. Parents had to be formally asked for their consent for the participation of their child in the SCID screening pilot by screeners. As the Centre for Population Screening monitors outcomes of the routine screening program, the close collaboration between the pilot study project group and CvB ensures the concurrent execution of the pilot study and the routine screening program.

Cost-Effectiveness Analysis of Newborn Screening for SCID

Cost-effectiveness studies for newborn screening for SCID have already been performed in the United States and New Zealand [14–16]. However, as costs and benefits of screening and treatment are likely to differ between countries and especially between continents, a cost-effectiveness analysis (CEA) for SCID was carried out by the Netherlands Organization for applied scientific research (TNO) in collaboration with Leiden University Medical Centre (LUMC). Lifetime costs and effects of newborn screening for SCID were compared with a situation without screening in the Netherlands in a decision analysis model. Model parameters were based on literature and expert opinions, after which sensitivity analyses were performed. The results lead to the publication of a report entitled "Cost-effectiveness and cost–benefit analysis (CEA/CBA) for SCID screening within the Dutch newborn screening program" in April 2017 [17, 18]. The SCID screening situation lead to additional costs for laboratory testing and follow-up diagnostics, but the costs of treatment of SCID patients were expected to decrease if newborn screening for SCID would be implemented. Although more patients would receive treatment in the form of hematopoietic stem cell transplantation, the early detection of the disease would result in lower transplantation-associated costs [19]. The long-term treatment costs would be lower as well, as early transplantation results in more favorable health outcomes. The results for the Netherlands are comparable with cost-effectiveness studies in the United States [14, 15] and indicate that SCID screening might be cost-effective, but the range of possible cost-effectiveness ratios is broad due to many parameter-associated uncertainties such as the incidence of SCID, costs of screening tests and costs of late transplantation. In conclusion, SCID screening in the Netherlands could be cost-effective but, due to many uncertainties, an extensive pilot study should be performed to help actualize the results of this CEA.

SCID Screening Assays Comparison Study

Prior to the prospective pilot study, a small-scale pilot study had already been performed in the Netherlands to show that the TREC assay is a suitable method for the Dutch newborn screening situation [20]. Since then, other newborn screening assays for SCID became commercially available and in order to select the most suitable assay for the large-scale prospective pilot study, an objective comparison study was performed. There were two commercially available newborn SCID screening assays at the time of the comparison study: the EnLite™. Neonatal TREC kit of PerkinElmer and the SCREEN-ID neonatal screening kit of ImmunoIVD (now called SPOT-it™ neonatal screening assay). Based on pre-set objective comparison criteria (established and approved before the evaluation phase by several parties including the CvB), the test qualities of both available SCID screening assays and their applicability for the Dutch screening situation were evaluated. The EnLite Neonatal TREC assay is a dried blood spot assay

employing PCR-based nucleic acid amplification and time-resolved fluorescence resonance energy transfer (TR-FRET) technology. The EnLite Neonatal TREC assay involves punching of dried blood spot specimens with a 1.5 mm punch head, adding elution buffer and starting elution incubation. After elution, reagent mixture is added and thermal incubation consisting of DNA amplification and probe hybridization is carried out. Signals from hybridized TREC and β-actin (internal control for monitoring amplification) probes are measured with the VICTOR™ EnLite Instrument (PerkinElmer). The assay is an in vitro diagnostic device intended for the semi-quantitative, multiplex determination of TREC and β-actin [21]. The SCREEN-ID assay is an in vitro diagnostic kit intended for routine screening of fresh, prospective neonatal dried blood spot samples. The assay is an all-in-one system for the quantitation of T-cell specific TRECs and/or B-cell specific KRECs as well as quality control marker β-actin using quantitative multiplex real-time PCR (qPCR). The SCREEN-ID assay employs regular 3.2 mm Guthrie card spots and features a Filter plate concept to rinse samples prior to analysis. The kit includes all necessary reagents pre-arranged in a set of Elution and qPCR plates and requires only two pipetting steps. As the triplex-detection chemistry for TRECs, KRECs and β-actin markers is independent of each analyte, the user can tailor the required diagnostic output of the SCREEN-ID assay. Some users might limit the screening approach to TRECs only, while others choose to report both TRECs and KRECs [22]. As the Dutch Health Council and Ministry of Health decided that newborn screening for SCID should be solely based on TREC detection, only the TRECs—and not the KRECs—detection feature of the assays were used and evaluated in this comparison study. To compare both SCID screening assays, 1272 anonymized fresh heel prick samples from the Dutch newborn screening program were analyzed. Moreover, peripheral blood from eight patients with a clinical, genetically confirmed SCID diagnosis (affected genes: RAG1, N = 3; RAG2, N = 2; IL2Rg, N = 1; XLF N = 1; Artemis N = 1) were included as well. Both assays were performed adhering strictly to the instructions of the manufacturer with the recommended instruments. Both manufacturers provided the researcher with personal training before performing the analyses. There were no deviations from either one of the protocols. The mean TREC level of 1272 anonymized Dutch heel prick cards was 123 copies/μL blood (median TREC: 102 copies/μL) for the EnLite Neonatal TREC assay and 116 copies/μL blood (median TREC: 109 copies/μL) for the SCREEN-ID assay (Table 1). The number of heel prick cards below the 2.5 percentile-mark was identical for both assays (N = 32). However, of these 32 heel prick cards, only eight cards presented with TREC levels below the 2.5 percentile in both assays. The remaining 24 heel prick cards showed disparate TREC levels due to poor amplification and low β-actin levels in either one of the assays. In the routine screening program, these samples would require retesting in duplicate. Retesting was not performed during the comparison study, as retest rates and referral rates based on this small sample size would not

be reliable comparison criteria. In this study, an experimental TREC cut-off level of 30 copies/µL was used for the EnLite Neonatal TREC assay to distinguish screen positive samples, based on advisory information of the manufacturer (PerkinElmer). For the SCREEN-ID kit an experimental TREC cut-off level of 6 copies/µl was used, based on advisory information of the manufacturer (ImmunoIVD). Both manufacturers recommend performing a large sample size pilot study to establish a preferred cut-off value based on the normal population distribution. With the TREC cut-off set at 30 copies/µL blood, 38 samples (3.0%) required a retest after the initial analysis with the EnLite Neonatal TREC assay. With the TREC cut-off set at 6 copies/µL blood, five samples (0.39%) required a retest after the initial analysis with the SCREEN-ID assay. As mentioned above, the number of samples below the cut-off value was not included as a comparison criterion, as each laboratory should establish a cut-off value based on a large sample size pilot study. The distribution of TREC levels in the analyzed heel prick cards is displayed in Figure 2. Samples of all eight genetically confirmed SCID patients had absent TREC levels, below the cut-off levels proposed in the respective kit-inserts.

Table 1. Results of the analysis of 1272 fresh heel prick cards. The average, median and 2.5 percentile of both severe combined immunodeficiency (SCID) screening assays are depicted in copies/µL blood. The number of heel prick cards with T-cell receptor excision circle (TREC) levels below the 2.5 percentile and the number below the cut-off of the manufacturer are also shown.

	EnLite Neonatal TREC Assay	**SCREEN-ID Assay**
Average (TREC copies/µL)	123	116
Median (TREC copies/µL)	102	109
2.5 percentile (TREC copies/µL)	28	33
Number of heel prick cards below 2.5 percentile	32	32
Number of heel prick cards below manufacturer's cut-off	38	5

The comparison criteria were subdivided into categories, each with a maximum amount of points to be awarded, namely Applicability (50 points), Analytical Procedure (205 points), Equipment and Software (65 points), Pricing (85 points) and Quality and Service (110 points). As the comparison criteria might be used for future tender procedures, the document cannot be made publicly available. Both SCID screening assays turned out to be suitable TREC detecting assays for the Dutch screening laboratories. Subtle differences lead to the selection of the assay with the most awarded overall points, namely the SCREEN-ID neonatal screening assay of ImmunoIVD. The SCREEN-ID assay is therefore used in the large scale implementation pilot study.

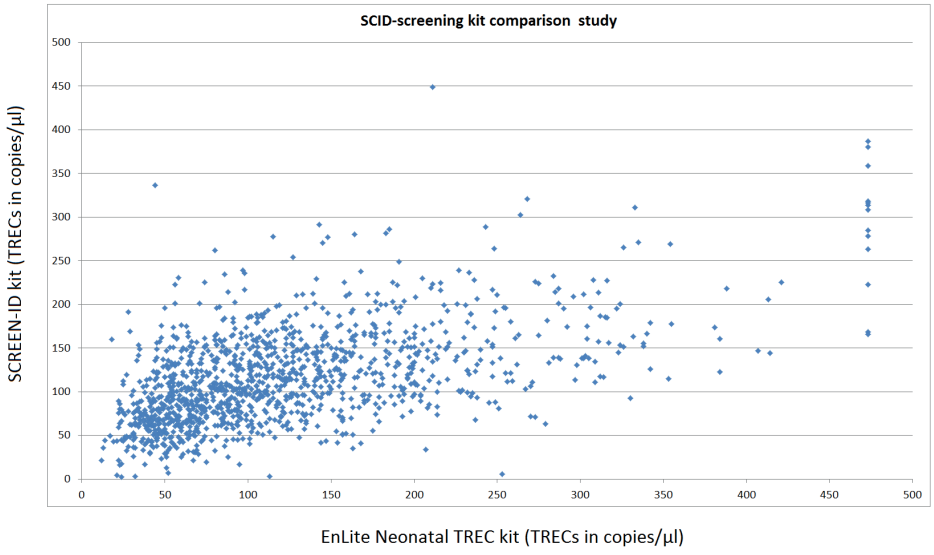

Figure 2. Comparison of TREC levels in 1272 heel prick cards analyzed with both SCID screening assays. Data in the diagram are displayed in a scatter-plot with EnLite Neonatal TREC analyses on the x-axis and SCREEN-ID analyses on the y-axis.

SCID Screening Prospective Implementation Pilot Study

As previously described, implementation of neonatal screening for SCID is complex due to expensive screening methods and intensive treatment options, such as hematopoietic stem cell transplantation. Moreover, there are a number of uncertainties, ranging from the expected number of referrals and analytical difficulties to unanticipated logistic challenges and unexpected screening outcomes. These uncertainties might seriously hamper the introduction of SCID screening in the routine program. In order to enable a flawless implementation of SCID screening, a prospective implementation pilot study within the routine screening program supported by The Netherlands Organisation for Health Research and Development (ZonMw) is being executed. ZonMw funds health research and promotes the use of the knowledge this research produces. The pilot study aims to gather knowledge about the practical implications of newborn screening for SCID, the cost-effectiveness, diagnostic and clinical follow-up issues, and the perspectives of health care providers and parents. This study will also assess the incidental findings accompanied by newborn screening for SCID, such as secondary T-lymphopenia due to congenital anomalies, or syndromes with T-cell impairment such as DiGeorge syndrome, trisomy 21, trisomy 18, and CHARGE syndrome [6]. As the Dutch Health Council has already deemed SCID a suitable candidate for the Dutch newborn screening program, that meets the Wilson and Jungner criteria [23], this pilot

study does not focus on whether the TREC assay is a suitable method for the detection of SCID. The effectiveness of newborn screening for SCID has already been proven in other screening programs abroad [6, 7], and previous research has provided us with a clear overview of the SCID disease in the Netherlands [24]. The implementation pilot will answer four main research questions: How can the TREC screening method be implemented in the current neonatal screening program? What are the test qualities of TREC detection in "real life" in the Netherlands? What are the costs for introduction of SCID screening in the neonatal screening program? How can adequate information and counselling facilitate an acceptable screening process for parents and their health care? The implementation pilot or SONNET study (SCID screening Research/Onderzoek in the Netherlands with TRECs) uses the infrastructure of the Dutch newborn screening program. The TREC assay is performed in two screening laboratories (RIVM in Bilthoven and IJsselland Hospital in Capelle aan den IJssel) and includes the newborns of three provinces (Utrecht, Gelderland and Zuid Holland). The pilot study started in April 2018 and includes the yearly workload of two screening laboratories, approximately 70,000 newborns. The project plan is based on four work packages that will be carried out over a two-year period. The first work package includes all preparatory steps required for the test phase that started on 1 April 2018. Information brochures for parents and health care providers have been distributed, informing them about SCID and the SCID screening pilot study. Parents receive information at different points in time allowing them to make an informed decision to participate in the pilot study. The information brochure contains information about the condition SCID, the goal and necessity of the implementation pilot, the advantages and disadvantages of participating, test results and privacy. Summaries of the brochure are available in English, Polish, Turkish and Arabic. Moreover, a website with additional information about the pilot and the latest development has been developed (www.sonnetstudie.nl). Informative meetings have been organized for screeners, midwifes, pediatricians and other parties involved in the pilot study. The screening laboratories have been adjusted and equipped for the PCR test method and technicians have received training from the manufacturer of the TREC assay. IT software has been updated, enabling the SCID screening results to be included in the routine screening databases. A flow chart for the screening laboratories has been designed (Figure 3), in which a distinction is made between full term and preterm infants (gestational age ≤ 36 weeks and birth weight ≤ 2500 g). Full term infants with an abnormal TREC result are referred to a pediatrician immunologist in one of the academic medical centers within 72 hours. Immunophenotyping by flow cytometry is the first step in the diagnostic follow-up. If T-lymphocytes are absent, genetic analysis (whole exome sequencing) with a pre-set SCID gene panel will be a secondary step performed by the academic medical center of referral. Newborns with absent T-cells will simultaneously start with the hematopoietic stem cell transplantation work-up. If

T-cells are low or non-functional, extra immunological diagnostics will be carried out in addition to the SCID gene panel analysis. Even though some of the incidental secondary findings of SCID screening are incurable, protective measures and prophylaxis could still provide a health gain for the individual. The follow-up procedure for SCID and non-SCID patients has been uniformly determined by pediatricians of all participating academic medical centers. The second work package focusses on the "real-life" SCID screening phase. TRECs are measured in approximately 70,000 newborns over a period of one year, with an interim evaluation after three months of screening. During the pilot study, KRECs are measured as well, but data are anonymized and used for research purposes only. In the third work package, the CEA of 2017 will be refined with new input data obtained from the prospective pilot study. The final work package focuses on the ethical, legal, and societal implications (ELSI) of newborn screening for SCID and the expansion of the newborn screening program. SCID is a case example of a disorder with high impact secondary findings and potential false positive rates, and the perspectives of parents and their health care providers are of great value. After the large scale pilot study, the Dutch Ministry of Health, Welfare and Sport will assess the evaluation report and make a final decision about the implementation of newborn screening for SCID in the Netherlands.

International Collaboration

At the end of 2017, the RIVM organized a first meeting about SCID screening with newborn screening experts from the Karolinksa Institutet (Stockholm, Sweden) and experts of the newborn screening program of the United Kingdom. In this meeting, new developments were discussed and experiences and results were shared, providing new inspiration for all parties involved. At the 11th European regional meeting of the International Society of Newborn Screening (ISNS) in Bratislava, Slovakia (October 2018), a session about newborn screening for SCID was organized in which experiences of several countries with regard to SCID screening were shared. Moreover, future developments in the field of newborn screening for primary immunodeficiencies, such as newborn screening for XLA, congenital neutropenia and IPEX syndrome were also discussed. As newborn screening for SCID in Europe is still in its infancy, with many European countries planning pilot studies or awaiting governmental implementation decisions, the RIVM is open for collaboration with all interested parties. Newborn screening for SCID might provide the perfect opportunity to initiate more international collaboration in the field of newborn screening.

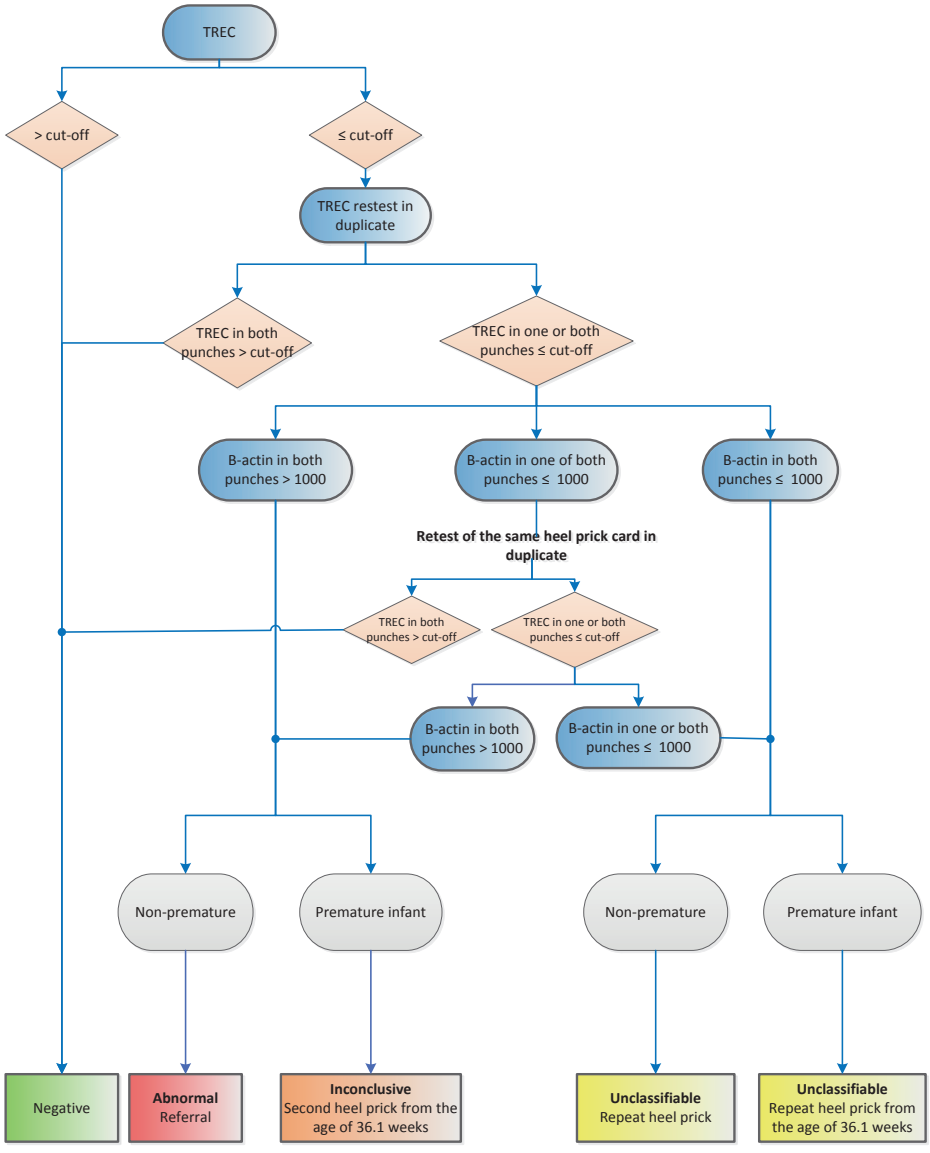

Figure 3. A flow chart of the TREC assay and the referral procedure. Premature infants are newborns with gestational age ≤ 36 weeks and birth weight ≤ 2500 g. TREC are T-cell receptor excision circles and ACTB is β-actin, the internal reference control.

Author Contributions

M.B. drafted the manuscript. E.H.B.M.D. is a member of the project group of the SONNET study and is the first author of the feasibility study by the Centre for Population Screening. E.A.K. and G.W. are members of the project group of the SONNET study and wield an advisory role. M.B. performed the experiments of the comparison study and analyzed the data. C.P.B.v.d.P. and E.v.d.A.-v.M. are members of the project group of the SONNET study and the authors of the cost-effectiveness analysis. M.v.d.B. is the project leader of the SONNET study and wrote the research proposal of the SONNET study with co-project leaders R.G.M.B. and P.C.J.I.S. All authors have reviewed and approved the final version of the manuscript.

Acknowledgments

We would like to thank all members of our guidance committee for their advice in the prospective pilot study (SONNET study): M.C van de Vries, M.E. van Gijn (UMCU), P.M. van Hasselt (UMC-WKZ), M.E. Jansen (RIVM), M. Jansen (RIVM), T.W. Kuijpers (AMC), A.J.J. Lock (RIVM), J. van Montfrans (UMC-WKZ), G.W.E. van Santen (LUMC), H. van Voorn (RIVM-DVP-West) and J. Verstegen (SAS).

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

1. van der Ploeg, C.P.B.; Wins, S.; Olthof, R.; Eekhout, I.; Verkerk, P.H. The newborn blood spot screening in the Netherlands—Monitor 2017,. TNO and Rijksinstituut voor Volksgezondheid en Milieu. 2018; report number TNO 2018 R11355.
2. Almannai, M.; Marom, R.; Sutton, V.R. Newborn screening: A review of history, recent advancements, and future perspectives in the era of next generation sequencing. Curr. Opin. Pediatr. 2016, 28, 694–699.
3. El-Hattab, A.W.; Almannai, M.; Sutton, V.R. Newborn screening: History, Current Status, and Future Directions. Pediatr. Clin. N. Am. 2018, 65, 389–405.
4. Baker, M.W.; Grossman, W.J.; Laessig, R.H.; Hoffman, G.L.; Brokopp, C.D.; Kurtycz, D.F.; Cogley, M.F.; Litsheim, T.J.; Katcher, M.L.; Routes, J.M. Development of a routine newborn screening protocol for severe combined immunodeficiency. J. Allergy Clin. Immunol. 2009, 124, 522–527.
5. Gerstel-Thompson, J.L.; Wilkey, J.F.; Baptiste, J.C.; Navas, J.S.; Pai, S.Y.; Pass, K.A.; Eaton, R.B.; Comeau, A.M. High-throughput multiplexed T-cell-receptor excision circle quantitative PCR assay with internal controls for detection of severe combined immunodeficiency in population-based newborn screening. Clin Chem. 2010, 56, 1466–1474.
6. Kwan, A.; Abraham, R.S.; Currier, R.; Brower, A.; Andruszewski, K.; Abbott, J.K.; Baker,M.; Ballow, M.; Bartoshesky, L.E.; Bonagura, V.R.; et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA. 2014, 20, 729–738.
7. Rechavi, E.; Lev, A.; Simon, A.J.; Stauber, T.; Daas, S.; Saraf-Levy, T.; Broides, A.; Nahum, A.; Marcus, N.; Hanna, S.; et al. First year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front. Immunol. 2017, 8, 1448.
8. Chien, Y.; Yu, H.; Lee, N.; Ho, H.; Kao, S.; Lu, M.; Jaing, T.; Lee, W.; Chang, K.; Shieh, C.; et al. Newborn Screening for Severe Combined Immunodeficiency in Taiwan. Int. J. Neonatal. Screen. 2017, 3, 16.
9. Health Council of the Netherlands. Neonatal Screening; Health Council of the Netherlands: The Hague, The Netherlands, 2005.
10. Health Council of the Netherlands. Neonatal Screening for Cystic Fibrosis; Health Council of the Netherlands: The Hague, The Netherlands, 2010.
11. Health Council of the Netherlands. Neonatal Screening: New Recommendations; Health Council of the Netherlands: The Hague, The Netherlands, 2015.
12. Letter of the Dutch Minister for Health (mw. drs. E.I. Schippers) to the Chairman of the House of Representatives of the Netherlands on the expansion of the newborn screening program. Available online: http://artsenjgz.nl/nieuwsbericht/reactie-minister-van-vws-op-advies-gezondheidsraad-over-14-nieuwe-aandoeningen-in-de-hielprikscreening/> (accessed on 28 May 2018).
13. Dekkers, E.H.B.M.; Klein, A.W.; Lock, A.J.J.; Vermeulen, H.M. Uitvoeringstoets uitbreiding neonatale hielprikscreening. Rijksinstituut voor Volksgezondheid en Milieu 2017.
14. Ding, Y.; Thompson, J.D.; Kobrynski, L.; Ojodu, J.; Zarbalian, G.; Grosse, S.D. Cost-Effectiveness/Cost-Benefit Analysis of Newborn Screening for Severe Combined Immune Deficiency in Washington State. J Pediatr. 2016, 172, 127–135.
15. Chan, K.; Davis, J.; Pai, S.Y.; Bonilla, F.A.; Puck, J.M.; Apkon, M. A Markov model to analyze costeffectiveness of screening for severe combined immunodeficiency (SCID). Mol. Genet. Metab. 2011, 104, 383–389.

16. Health Partners Consulting Group. Cost-effectiveness of newborn screening for Severe Combined Immune Deficiency. A Report prepared for the National Screening Unit. Available online: https://www.nsu.govt.nz/system/files/resources/cost-effectiveness-newborn-screening-severe-combined-immune-deficiency.pdf (accessed on 20 November 2018).
17. van der Ploeg, C.P.B.; van den Akker-van Marle, E.; Bredius, R.G.M.; Staal, F.; van den Burg, M.; Verkerk, P. Kosteneffectiviteits- en kostenbatenanalyse (KEA/KBA) voor het screenen op SCID binnen de Nederlandse hielprikscreening. TNO 2017.
18. Van der Ploeg, C.P.B.; Blom, M.; Bredius, R.G.M.; van der Burg, M.; Schielen, P.C.J.I.; Verkerk, P.H.; van den Akker-van Marle, M.E. Cost-effectiveness of newborn screening for severe combined immunodeficiency. Eur. J. Pediatrics 20191. 78(5), 721-729.
19. Clément, M.C.; Mahlaoui N.; Mignot C.; le Bihan C.; Rabetrano H.; Hoang L.; Neven B.; Moshous D.; Cavazzana M.; Blanche S.; et al. Systematic neonatal screening for severe combined immunodeficiency and severe T-cell lymphopenia: Analysis of cost-effectiveness based on French real field data. J. Allergy Clin. Immunol. 2015, 135, 1589–1593.
20. Blom, M.; Pico-Knijnenburg, I.; Sijne-van Veen, M.; Boelen, A.; Bredius, R.G.M.; van der Brug, M.; Schielen, P.J.C.I. An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newbon screening program. Clin. Immunol. 2017.
21. PerkinElmer. EnLite Neonatal TREC kit. Available online: https://newbornscreening.perkinelmer.com/products/enlite_neonatal_trec_instrument/enlite_neonatal_trec_kit (accessed on 25 May 2018).
22. ImmunoIVD. SCREEN-ID neonatal screening kit. Available online: http://immunoivd.com/technology.html (accessed on 24 May 2018).
23. Wilson, J.M.G.; Jungner, G. Principles and practice of screening for disease. Geneva: WHO; 1968. Available online: http://www.who.int/bulletin/volumes/86/4/07-050112BP.pdf (accessed on 18 May 2018).
24. De Pagter, A.P.J.; Bredius, R.G.M.; Kuijpers, T.W.; Tramper, J.; van der Burg, M.; van Montfrans, J.; Driessen, G.J. Overview of 15-year severe combined immunodeficiency in the Netherlands: Towards newborn blood spot screening. Eur J Pediatr. 2015, 174, 1183–1188,.

CHAPTER 4

Parents' perspectives and societal acceptance of implementation of newborn screening for SCID in the Netherlands

Maartje Blom, Robbert G. M. Bredius, Marleen E. Jansen, Gert Weijman, Evelien A. Kemper, Clementien L. Vermont, Iris H. I. M. Hollink, Willem A. Dik, Joris M. van Montfrans, Mariëlle E. van Gijn, Stefanie S. Henriet, Koen J. van Aerde, Wouter Koole, Arjan C. Lankester, Eugènie H. B. M. Dekkers, Peter C. J. I. Schielen, Martine C. de Vries, Lidewij Henneman and Mirjam van der Burg on behalf of the SONNET-Study Group

Journal of Clinical Immunology, 2021; 41: 99-108

ABSTRACT

Purpose

While neonatal bloodspot screening (NBS) for severe combined immunodeficiency (SCID) has been introduced more than a decade ago, implementation in NBS programs remains challenging in many countries. Even if high-quality test methods and follow-up care are available, public uptake and parental acceptance are not guaranteed. The aim of this study was to describe the parental perspective on NBS for SCID in the context of an implementation pilot. Psychosocial aspects have never been studied before for NBS for SCID and are important for societal acceptance, a major criterion when introducing new disorders in NBS programs.

Methods

To evaluate the perspective of parents, interviews were conducted with parents of newborns with abnormal SCID screening results (N = 17). In addition, questionnaires about NBS for SCID were sent to 2,000 parents of healthy newborns who either participated or declined participation in the SONNET-study that screened 140,593 newborns for SCID.

Results

Support for NBS for SCID was expressed by the majority of parents in questionnaires from both a public health perspective as a personal perspective. Parents emphasized the emotional impact of an abnormal screening result in interviews. (Long-term) stress and anxiety can be experienced during and after referral indicating the importance of uniform follow-up protocols and adequate information provision.

Conclusion

The perspective of parents has led to several recommendations for NBS programs that are considering screening for SCID or other disorders. A close partnership of NBS programs' stakeholders, immunologists, geneticists and pediatricians-immunologists in different countries is required for moving towards universal SCID screening for all infants.

INTRODUCTION

In the past decade, neonatal bloodspot screening (NBS) for severe combined immunodeficiency (SCID) has been introduced in several screening programs worldwide [1-5]. After addition to the Recommended Uniform Screening Panel (RUSP) in the USA, all states introduced SCID screening progressively, realizing nationwide screening for SCID in 2018 [6]. Even though the screening technique for SCID has been available for over a decade, implementation into screening programs is accompanied by many challenges due to the complexity of NBS programs. NBS encompasses more than a laboratory test and implementation includes adjustments in education, finances, logistics, politics and culture [7-9] and even if a high-quality test method is available, public uptake and parental acceptance of the test method are not guaranteed.

SCID is one of the most severe inherited disorders of the immune system characterized by severe T-cell lymphopenia that is variably associated with an abnormal development of B- and/or natural killer (NK)-cells [10]. Patients with SCID are usually born asymptomatic but develop life-threatening infections in the first months of life. Prompt clinical intervention with hematopoietic stem cell transplantation (HSCT) or gene therapy is required to prevent a fatal outcome for these patients [11]. Previous studies showed that early detection and treatment in the pre-symptomatic phase lead to higher survival rates [12-14]. NBS for SCID is based on the measurement of T-cell receptor excision circles (TRECs) via (semi-)quantitative PCR. TRECs are circular DNA fragments formed during the T-cell receptor gene rearrangement in the thymus serving as a marker for thymic output [15]. Low TREC levels indicate reduced numbers of recently formed T-lymphocytes [16, 17]. To distinguish SCID from other T-cell lymphopenias, follow-up diagnostics by flow cytometric immunophenotyping and genetic analysis are indicated [18].

Similar to other countries [19-23], the Netherlands started a prospective implementation pilot study (SONNET-study) in April 2018, focusing on parental perspective, cost-effectiveness and practical implications for screening, diagnostics and clinical follow-up. As parents are important stakeholders in NBS, their support is paramount. NBS pilot studies provide an invaluable opportunity to assess parental views on the potential benefits and harms of screening for newborns and their families [24]. In many cases, experts will assume that patients and families will automatically welcome perceived advances in the field. However, this is not necessarily the case and it is important to gauge families perceptions of these advantages. Therefore, we investigated the societal and psychosocial aspects through the eyes of parents

of healthy newborns and parents who received an abnormal SCID screening result for their newborn. Our findings have led to important recommendations that can be valuable to other countries that consider implementation of SCID screening in their NBS program.

METHODS

For the SONNET-study, all parents of newborns born in three of the eleven provinces of the Netherlands (Utrecht, Gelderland and Zuid-Holland) were asked to participate in a research project on NBS for SCID (opt-out consent). All dried blood spots (DBS) included (N = 140,593) were collected as part of the Dutch routine NBS program from April 2018 to February 2020 (Figure S1). Demographic and clinical variables were collected from the national Praeventis NBS database (RIVM, Bilthoven, the Netherlands). The SONNET-study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146). TREC analysis was performed according to the SPOT-itTM kit instructions for use (ImmunoIVD, Stockholm, Sweden) according to a preset screening algorithm (Figure S2). From April 2018 to October 2018, a TREC cut-off value of ≤ 6 copies/3.2 mm punch was used. After six months of screening, the cut-off value was increased to ≤ 10 copies/3.2 mm punch from November 2018 to February 2020. A uniform diagnostic follow-up protocol and gene panel after abnormal TREC results was established (Figure S3 and Table S1). Interviews were conducted with parents after an abnormal SCID screening result (N = 17). Items in the interview were evaluated either by categorical or non-categorical variables, the latter through open questions that were independently keyword-coded by two researchers to enhance the internal validity of the results. The perspective of parents of healthy newborns on NBS for SCID who either participated (N = 1,600) or declined participation (N = 400) in the SONNET-study was evaluated with a questionnaire that was specifically developed for this study by a multidisciplinary team of experts on NBS, medical ethics, and survey studies. The questionnaire was based on existing questionnaires previously used for investigating parents' perspectives on NBS e.g. for Pompe disease [25]. For qualitive validation and to address educational and language barriers, a small test phase was conducted to check for concept and wording of questions. The final concept was peer-reviewed before sending out. Construct validation questions were not included as it was not the goal to create a quantitative validated questionnaire about NBS for SCID. Technical barriers were addressed by offering parents the opportunity to send back a printed questionnaire or to fill in the questionnaire online by following a link or scanning a QR-code. Multiple multivariate logistic regression analyses were performed to determine whether variables such as age, ethnicity and educational level induced bias. For further details, see Methods in Supplemental data.

RESULTS

TREC screening and Referrals

141,343 newborns participated in routine NBS in the pilot region. 750 parents of newborns declined participation in the SONNET-study (participation rate 99.5%). Median TREC level in the study population was 97 cop/3.2 mm punch (IQR 66-141; Table S2). Receiving a blood transfusion less than 24 hours prior to sample collection or early sample collection (< 72 hours after birth) resulted in lower TREC levels (P <0.05; Table S2). 333 of the 140,593 newborns had TREC levels below the preset cut-off value after initial analysis (retest rate 0.24%; Figure 1). In total, 47 full-term newborns with low TREC levels were referred for additional diagnostics (referral rate 0.03%; Figure 1).

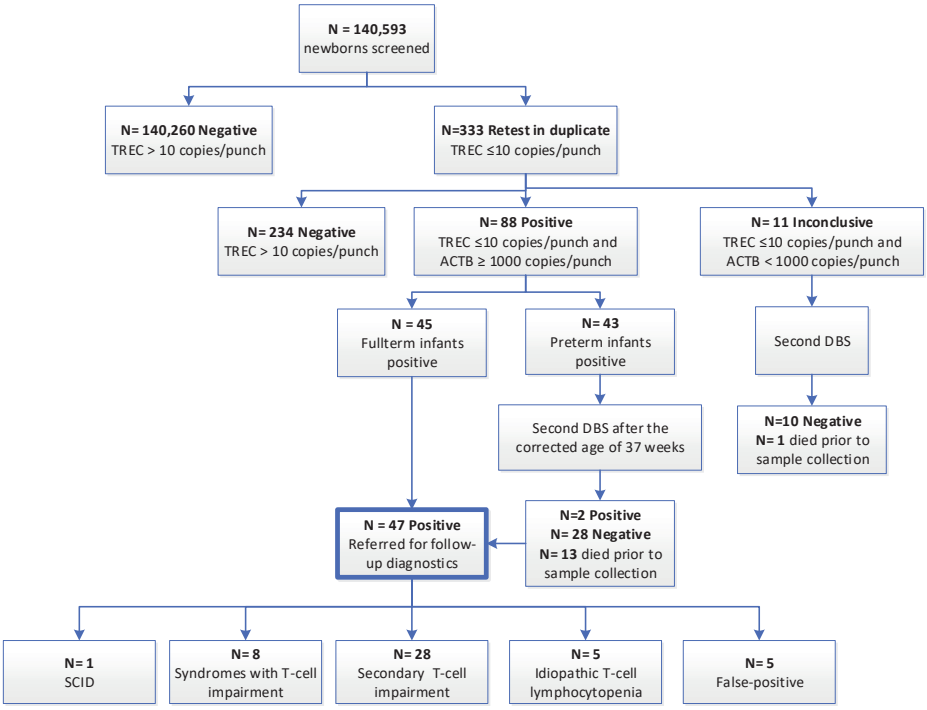

Figure 1. Number of referrals and retests based on TREC analysis. 140,593 newborns were included for initial TREC analysis. NBS cards with TREC ≤10 copies/3.2 mm punch required repeated analysis in duplicate. Preterm: age <37 weeks and birth weight ≤2500 grams. Abnormal screening results with β-actin (ACTB) levels less than 1000 copies/3.2 mm punch were considered inconclusive and required repeated sampling (second DBS).

One SCID patient was identified with absent TRECs (0 copies/3.2 mm punch) and absent T-cells. Genetic analysis revealed a pathogenic variant in the IL2RG gene (NM_000206.2(IL2RG):c.298C>T, p.(Gln100*)). The patient remained asymptomatic, underwent HSCT and is currently in good clinical condition. In the other 46 newborns referred for further evaluation, five newborns had normal flow cytometric results with no known underlying cause for the low TREC levels (false-positive cases; Figure 1). Of the 41 newborns with non-SCID T-cell lymphocytopenia (TCL), eight infants had a congenital syndrome associated with T-cell impairment, while five infants were reported to have idiopathic T-cell lymphocytopenia with an unknown underlying cause (Table S3). In 28 cases T-cell lymphopenia could be attributed to other medical conditions without an intrinsic defect in the production of T-cells (secondary T-cell impairment; Table S3).

Parents' experiences after an abnormal SCID screening result

The parents of 23 newborns referred with an abnormal SCID screening result were approached for an interview and 17/23 parents agreed (Table S4). Parents of eight newborns remembered receiving information about NBS for SCID prior to the heel prick and knowingly participated in the SCID pilot study. Nine parents did not remember receiving information and one mother even questioned whether she would have participated in the SCID pilot study if she would have been formally asked.

Fifteen newborns were referred via the general practitioner (GP) to an academic medical center, while two newborns were already in the hospital at the time of referral. Referral via the GP is the standard procedure in the Dutch NBS program (Figure S1). Parents of twelve newborns experienced the referral procedure as negative, stating that they either received too little or incorrect information via the GP. In addition, parents experienced the initial counselling by the GP as unpleasant, for example rushed via telephone contact instead of in person. Parents would have preferred to be contacted by a pediatric-immunologist directly so they could receive correct and clear information from the start with the opportunity to ask questions. One couple appreciated being called by a familiar and trusted person as their GP, whereas two mothers who received the news via telephone stated that a personal visit from the GP would be exorbitant.

The majority of parents (15/17) were very satisfied with the rapid availability of the diagnostic results and the follow-up care provided by the pediatric-immunologist. All parents stated to have experienced significant anxiety and emotional insecurity up to the visit in the hospital, however their trust in the NBS program had not been changed by this experience.

Parental perception of the vulnerability of their newborn after definitive diagnosis was determined with the Vulnerable Baby Score (VBS) (N = 13).The mean VBS was 28.7 (SD4.8) compared to 23.1 (SD3.1) as found in case of healthy control newborns [26] (Table 1). The mean total score of the parental stress questionnaire (OBVL) of these parents was 60.5 (SD 8.3) which is just above the norm for parents of children age category 0-3 years (Table 2). Parents experienced mild problems in the subcategory 'restrictions to one's own freedom and frustration in attempts to maintain one's own identity' (T-score of 65.1) (Table 2).

Table 1. Vulnerable Baby Score (VBS) by parents of newborns with abnormal SCID screening results (N = 13).

	N	Baby age when questionnaire completed in weeks Mean (range)	Mean VBS	SD
Healthy newborns	39	13.4 (11.2-17.3)	23.1	3.1
Jaundice	19	10.6 (9.6-14.1)	25.1	4.2
Medically fragile	17	11.4 (9.5–15.0)	27.4	4.6
Newborns with abnormal SCID screening results (total)	13	21.2 (8.5-41.7)	28.7	4.8
T-cell impairment syndromes	3		31.0	
Secondary T-cell impairment	3		28.3	
Idiopathic lymphocytopenia	4		30.7	
False positive	3		25.7	

Data of medically fragile, jaundice and healthy control groups adopted from Kerruish *et al.* [26].

Parental perspective on NBS for SCID and scientific research on NBS

391 of 2,000 parents of healthy newborns returned the questionnaire (response rate 19.6%). 84.9% (332/391) of parents participated in the SONNET-study. Sixteen (4.1%) parents declined participation and 33 (8.4%) parents could not remember whether they participated or not. The respondents' characteristics are shown in Table S5 and Table S6. The mean age of respondents was 32.8 years (range 20–52 years). Most respondents were female (85.8%). Compared to the reference population (Table S5), respondents were higher educated and more likely to have a Dutch background.

Table 2. Parental stress scores (OBVL) by parents of newborns with abnormal SCID screening results (N = 13).

	N	Parent-child relationship problems	Parenting problems
T-cell impairment syndromes	3	62.7	63.7
Secondary T-cell impairment	3	54.3	49.3
Idiopathic lymphocytopenia	4	57	58.8
False positive	3	54.3	50.3
Total	13	57.1	55.8

T-scores are the transformed raw data scores. Mild problems are implied with T-scores above 65 for the subcategories and a T-score above 60 for the total score.

Table 3. Different information sources in the SCID pilot study and the evaluation scores of the received information by parents (N = 391)

Did you receive:	**Yes**	**No**	**I do not remember or I do not know**
	N (%)	**N (%)**	**N (%)**
Oral information via the midwife/gynecologist	107 (28.1)	195 (51.2)	79 (20.7)
Oral information via the screener	181 (47.5)	114 (29.9)	86 (22.6)
Information leaflet			
From midwife/gynecologist	64 (18.2)	212 (60.4)	75 (21.4)
From screener	59 (17.7)	202 (60.5)	73 (21.9)
At city hall	22 (6.8)	230 (71.4)	70 (21.7)
Visited the study website www.sonnetstudie.nl	13 (3.4)	368 (96.4)	1 (0.3)

Missing values were excluded from the percentages. Evaluation scores were not individually calculated for parents who could not remember whether they participated in the SCID pilot study (N = 33). *Mann-Whitney U-test.

Table 4. Difference in attitude of parents who participated or declined participation in SCID screening (N = 348)

Questionnaire statement
'Scientific research' related statements
Scientific research is required to prevent diseases
Scientific research is required to improve treatment of diseases
I do not want to participate in scientific research

Depressive mood	Parental role restriction	Physical Health problems	Total score
63	64	68.3	68.7
51.3	55.3	57	50.7
60.8	71.2	65.8	63.5
58.7	67.3	56.7	58
58.6	65.1	62.2	60.5

Evaluation score 1-10 (SD, N)			P-value*
Total	Participated in SCID pilot study	Declined participation in the SCID pilot study	
7.5 (SD 1.3, N = 128)	7.4 (SD 1.3; N = 118)	7.1 (SD 1.2; N = 6)	P = 0.537
7.1 (SD 1.5, N = 196)	7.2 (SD 1.4; N = 175)	5.7 (SD 1.4; N = 12)	P = 0.001
7.6 (SD 1.3, N = 112)	7.6 (SD 1.3; N = 110)	6.7 (SD 0.76; N = 7)	P = 0.019
7.8 (SD 1.6, N = 19)			

Participated (N = 332)		Declined (N = 16)		P-value*
Rating mean (SD)[a]	% that agreed	Rating mean (SD)[a]	% that agreed	
4.7 (0.64)	98.8	3.5 (1.41)	75	P < 0.01
4.7 (0.66)	98.2	3.9 (1.09)	87.5	P < 0.01
1.5 (0.79)	10.7	3.2 (1.11)	75	P < 0.01

Table 4. Continued

Questionnaire statement
'NBS for SCID' related statements
SCID is a severe disorder and I want this disorder to be detected in my child as early as possible
I think it is important that SCID is included in the newborn screening program
The person who performed the heel prick advised me to participate in SCID screening
My family / partner wanted the SCID test to be performed for my child
I only want my child tested for SCID once the study has been completed and SCID has been included in the newborn screening program
'Health of their child' related statements
I want as much information as possible about my child's health
I want to be reassured that my child does not have SCID
I do not worry about the health of my child
I think I have a high risk of getting a child with SCID

[a] Five-point rating scale: 1 = fully disagree; 5 = fully agree. SD = Standard deviation. Parents who could not remember whether they declined or participated (N = 33) and missing values (N = 10) are excluded from the percentages. *Mann-Whitney U-test

Respondents in the questionnaire study were orally informed about NBS for SCID by the midwife/gynecologist (N = 107; 28.1%) and/or the screener (N = 181; 47.5%) (Table 3). Information provision by the midwife/gynecologist was rated best (evaluation score of 7.5). The majority of parents did not recollect to have received or did not read the information leaflet (N = 272; 72%). Parents who did receive the information leaflet were positive (evaluation score of 7.6). These parents indicated that the leaflet was clear (N = 98; 87.5%) and easy to read (N = 90; 80.3%), and that information was sufficient and understandable (Figure S4).

Parents who declined participation in the SONNET-study were less positive about the provided information compared to parents who participated (Table 3). Participants were more likely to answer one of the knowledge questions correctly compared to parents who declined participation (P = 0.03) (Table S7).

Support for NBS for SCID was expressed by the majority of parents from a public health perspective "I think it is important that SCID is included in the newborn screening program" (rating mean 4.3) and a personal perspective "SCID is a severe disorder and I want this disorder to be detected as early as possible for my child" (rating mean 4.2;

Participated (N = 332)		Declined (N = 16)		P-value*
Rating mean (SD)[a]	% that agreed	Rating mean (SD)[a]	% that agreed	
4.4 (0.76)	98.2	2.9 (1.03)	62.5	P < 0.01
4.3 (0.74)	99.4	2.9 (0.93)	75	P < 0.01
1.5 (0.81)	14.2	1.3 (0.88)	0	P = 0.542
2.8 (1.24)	66.6	1.8 (1.13)	25	P < 0.01
2.1 (1.11)	26.5	2.6 (0.96)	56.2	P = 0.025
4.1 (0.90)	94.5	2.9 (1.36)	56.2	P < 0.01
4.0 (1.00)	91.5	2.7 (1.45)	50	P < 0.01
3.4 (1.19)	76.1	3.9 (1.24)	82.2	P = 0.047
1.8 (0.88)	26.9	1.4 (0.73)	12.5	P = 0.068

Table S8). Parents who declined participation in SCID screening had a more negative attitude towards scientific research in general (rating mean 3.5 versus 4.7 P <0.01) and believed it to be of less importance that SCID is included in the NBS program (rating mean 2.9 versus 4.3 P <0.01) (Table 4).

Reasons to participate or decline participation in NBS for SCID

Reasons to participate in NBS for SCID included the potential health benefit for their child (41.8%), to support scientific research (41.8%), the fact that no extra blood had to be drawn (12.5%), the disorder can be cured (8.1%) and to help other children (6.6%) (N = 340). Parents who declined participation (N = 16) stated they declined because of insufficient/misconception of information, a low a priori risk of the disease, the test still being in a research phase, not being interested in knowing or due to privacy reasons. Parents who read the leaflet/received information about the pilot study were not more likely to participate, but parents with higher knowledge scores were marginally more likely to participate in NBS for SCID (P = 0.06; Table S9). Respondents with one child (first-time parents) were more likely to participate in NBS for SCID compared to parents with more children (P = 0.04; Table S9).

DISCUSSION

NBS for SCID based on TREC quantification has been implemented in several countries, thus the effectiveness of TREC quantification for SCID detection has been demonstrated [27, 28]. However, the availability of a high-quality test method does not automatically guarantees acceptance from the perspective of stakeholders such as parents. Therefore, our study focused on societal context including public awareness and understanding by studying the perspectives of parents and evaluating the practical aspects for screening, diagnostic procedures and clinical follow-up. Psychosocial aspects have never been reported before in NBS for SCID while they are important for societal acceptance, a major criterion when introducing new disorders in NBS programs.

Interviews with parents revealed that parents experienced anxiety and stress when receiving an abnormal screening result for SCID. Most parents were informed by their GP and felt their GP lacked important knowledge about SCID while experiencing telephone contact as impersonal and rushed. International studies show that health care providers acknowledge the difficulty of delivering abnormal screening results to parents [29, 30]. Some providers deliberately keep information during this first contact to a minimum trying to reduce parental anxiety [31]. Communication scripts developed together with parents could help a primary health care provider in this first contact [29]. In the interviews, parents suggested tandem telephone calls with both their GP and a pediatric-immunologist to provide support and expert information at the time of the referral. Most parents commended their experience with the pediatric-immunologist and were relieved with the rapid availability of diagnostic results. The magnitude of parents' distress while waiting for infants' confirmatory test results should not be underestimated [30]. Similar to studies for NBS for cystic fibrosis (CF), all parents would still participate in NBS for SCID despite their experiences in the referral procedure [32-34]. Parents scored relatively high on the Vulnerable Baby Scale in comparison to parents of healthy newborns [26]. Even parents with a confirmed healthy newborn after follow-up (false-positive) perceived their newborn as more 'vulnerable' implying some effect of the referral procedure with the associated feelings of anxiety [35, 36]. Parents additionally experienced some mild problems in their parental role. The interviews provide a more in-depth understanding of the impact of an abnormal SCID screening result for parents and emphasize the importance of reducing false-positive referrals.

Our questionnaire study amongst parents of healthy newborns showed that parents have a positive attitude towards NBS for SCID. Most parents stated that they wanted SCID to be detected as early as possible for their child. While our respondent group was different from the Dutch reference population, their opinion might still reflect the attitude of the general Dutch population. Other studies have also shown public support for expanded NBS and a positive attitude towards NBS in general [37-39]. As these studies also used self-developed surveys, one could argue that there is a need for a general validated questionnaire that evaluates parental perspectives on implementation of new disorders in NBS programs. First-time parents with only one child were more likely to participate in NBS for SCID than parents with more children. These findings were also observed in our previous questionnaire study in which 'new' parents were more likely to participate in hypothetical NBS for the untreatable disorder ataxia telangiectasia, a potential incidental finding for NBS for SCID [40, 41]. The key motivator for parents for participation in NBS for SCID was to benefit the health of their own child, but also supporting scientific research and the non-invasive character of NBS for SCID were reported reasons. These findings are in accordance with previous studies in which reasons for accepting newborn screening were investigated [42-44].

Some parents declined participation in NBS for SCID due to insufficient information and misconception of the pilot study, illustrating the importance of providing adequate information in NBS programs. Our findings confirm previous research indicating that NBS education does not always reach parents and there is a persistent lack of public knowledge about NBS [37, 45]. These studies also showed that healthcare providers are the preferred source of NBS information, advocating for incorporation of NBS education into prenatal care and for midwifes to counsel parents [37, 45]. Information provision and timing of information in NBS has been an ongoing topic of discussion with little consensus between countries [46]. Other means such as digital apps or videos should be explored in the near future.

In summary, our pilot study shows that while the central idea of early detection of SCID to facilitate treatment is simple, successful implementation of NBS for SCID is a complex process with parental acceptance being of great importance when introducing new disorders in NBS programs. The findings of this study on parental perspectives have led to several recommendations for other NBS programs that are considering SCID screening or future implementation of other disorders (Figure 2).

Recommendations

- Clear information provision by the indicated health care provider both prior to the NBS program as well as during the referral procedure after an abnormal screening result is of utmost importance for parents.
- Tandem telephone calls by primary health care providers and pediatricians(-immunologists) should be considered when delivering the news about abnormal screening results to parents.
- (Long-term) stress and anxiety levels of parents after an abnormal screening result, independent of the outcome/diagnosis, should not be underestimated. Follow-up care/contact persons should be made available.
- All possible adaptations to the NBS leading to more targeted screening for the core condition SCID and the reduction of the number of incidental findings and false-positive cases should be explored.
- Uniform follow-up protocols are required for a prompt and consistent approach to a definitive diagnosis and can provide guidance for pediatrician-immunologists when dealing with the relatively high number of incidental findings accompanied by NBS for SCID.
- Parents' perspectives should be taken into account when introducing new disorders in NBS programs as societal acceptance is of utmost importance.
- A close partnership of NBS programs, patient organizations, immunologists, geneticists and HSCT specialists in different countries could help to promote standardization of care and follow-up protocols.
- Shared learning should be facilitated internationally to support effective implementation of SCID screening suited to the local context to move towards universal harmonized SCID screening for all infants.

Figure 2. Recommendation box. The findings of this study have led to several recommendations for other NBS programs that are considering SCID screening or future implementation of other disorders

Acknowledgments

The authors would like to thank all those involved in the SONNET-study for their contribution and support. We want to thank all stakeholders in the Dutch newborn screening program with a warm thanks to all parents participating in the SONNET-study, the questionnaire study and the interviews. The authors would like to express their gratitude to Myrthe Hulst for her aid in the questionnaire study and interviews, to all technicians from the RIVM and IJsselland hospital for all TREC-analyses and to the technicians from the participating academic medical centers for their aid in follow-up diagnostics. Finally, a special thanks to José Verstegen and the Patient Organization 'Stichting voor Afweerstoornissen (SAS)' for their involvement and support throughout the entire project.

Authorship Contributions
MvdB, RB, PS and ED designed the study; MB, EK, IH, WD, MG and WK performed analyses; MB, MJ, MV and LH designed and performed questionnaire study; RB, GW, CV, JM, SH, KA and AL did the clinical evaluations; MB and MJ analyzed the data; MvdB coordinated the project; MB, LH and MvdB wrote the paper; all authors contributed to and approved the final version of the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of Conflicts of Interest

Funding
This study was funded by The Netherlands Organisation for Health Research and Development ZonMW (SONNET study, project 543002002). LH received funding from ZonMw to study the psychosocial aspects of (expanded) NBS (PANDA study, project 543002006).

Conflict of Interest
The authors declare that they have no conflict of interest.

Declarations

Ethics approval
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

Consent to participate
In order to participate in the SONNET-study, parents have to express verbal consent when the heel prick is performed (opt-out consent). Filling out the questionnaire was voluntary and participation after receiving the invitation implied consent.

REFERENCES

1. Kwan, A., et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama, 2014. 312(7): p. 729-38.
2. Chien, Y.-H., et al., Newborn Screening for Severe Combined Immunodeficiency in Taiwan. International Journal of Neonatal Screening, 2017. 3(3): p. 16.
3. Rechavi, E., et al., Newborn Screening for Severe Combined Immunodeficiency in Israel. International Journal of Neonatal Screening, 2017. 3(2): p. 13.
4. van der Burg, M., et al., Universal Newborn Screening for Severe Combined Immunodeficiency (SCID). Frontiers in pediatrics, 2019. 7: p. 373-373.
5. Argudo-Ramírez, A., et al., First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain). Frontiers in immunology, 2019. 10: p. 2406-2406.
6. Routes, J. and J. Verbsky, Newborn Screening for Severe Combined Immunodeficiency. Curr Allergy Asthma Rep, 2018. 18(6): p. 34.
7. Jansen, M.E., et al., Policy Making in Newborn Screening Needs a Structured and Transparent Approach. Frontiers in Public Health, 2017. 5(53).
8. Therrell, B.L., U.S. Newborn Screening Policy Dilemmas for the Twenty-First Century. Molecular Genetics and Metabolism, 2001. 74(1): p. 64-74.
9. Dhondt, J.-L., Expanded newborn screening: social and ethical issues. Journal of Inherited Metabolic Disease, 2010. 33(S2): p. 211-217.
10. Picard, C., et al., International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity. J Clin Immunol, 2018. 38(1): p. 96-128.
11. Fischer, A., et al., Severe combined immunodeficiencies and related disorders. Nat Rev Dis Primers, 2015. 1: p. 15061.
12. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
13. Pai, S.Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med, 2014. 371(5): p. 434-46.
14. Brown, L., et al., Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. Blood, 2011. 117(11): p. 3243-6.
15. Hazenberg, M.D., et al., T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J Mol Med (Berl), 2001. 79(11): p. 631-40.
16. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2).
17. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
18. Kalina, T., et al., EuroFlow Standardized Approach to Diagnostic Immunopheneotyping of Severe PID in Newborns and Young Children. Front Immunol, 2020. 11: p. 371.
19. Audrain, M.A.P., et al., Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). J Clin Immunol, 2018. 38(7): p. 778-786.

20. Blom, M., et al., An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. Clin Immunol, 2017. 180: p. 106-110.
21. Can, C., S. Hamilcikan, and E. Can, Early diagnosis of severe combined immunodeficiency (SCID) in Turkey: a pilot study. J Matern Fetal Neonatal Med, 2018. 31(24): p. 3238-3242.
22. Zetterström, R.H., et al., Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden. International Journal of Neonatal Screening, 2017. 3(2): p. 11.
23. Kanegae, M.P.P., et al., Newborn Screening for Severe Combined Immunodeficiencies Using TRECs and KRECs: Second Pilot Study in Brazil. Rev Paul Pediatr, 2017. 35(1): p. 25-32.
24. Goldenberg, A.J., et al., Including ELSI research questions in newborn screening pilot studies. Genetics in Medicine, 2019. 21(3): p. 525-533.
25. Weinreich, S.S., et al., Public support for neonatal screening for Pompe disease, a broad-phenotype condition. Orphanet J Rare Dis, 2012. 7: p. 15.
26. Kerruish, N.J., et al., Vulnerable Baby Scale: development and piloting of a questionnaire to measure maternal perceptions of their baby's vulnerability. J Paediatr Child Health, 2005. 41(8): p. 419-23.
27. Verbsky, J., M. Thakar, and J. Routes, The Wisconsin approach to newborn screening for severe combined immunodeficiency. The Journal of allergy and clinical immunology, 2012. 129(3): p. 622-627.
28. Hale, J.E., et al., Identification of an infant with severe combined immunodeficiency by newborn screening. The Journal of allergy and clinical immunology, 2010. 126(5): p. 1073-1074.
29. Moody, L., et al., Healthcare professionals' and parents' experiences of the confirmatory testing period: a qualitative study of the UK expanded newborn screening pilot. BMC pediatrics, 2017. 17(1): p. 121-121.
30. DeLuca, J.M., et al., Parents' Experiences of Expanded Newborn Screening Evaluations. Pediatrics, 2011. 128(1): p. 53-61.
31. Rueegg, C.S., et al., Newborn screening for cystic fibrosis - The parent perspective. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society, 2016. 15(4): p. 443-451.
32. Tluczek, A., et al., Psychological impact of false-positive results when screening for cystic fibrosis. Pediatric pulmonology. Supplement, 1991. 7: p. 29-37.
33. Tluczek, A., K.M. Orland, and L. Cavanagh, Psychosocial consequences of false-positive newborn screens for cystic fibrosis. Qualitative health research, 2011. 21(2): p. 174-186.
34. Vernooij-van Langen, A.M.M., et al., Parental knowledge reduces long term anxiety induced by false-positive test results after newborn screening for cystic fibrosis. Molecular genetics and metabolism reports, 2014. 1: p. 334-344.
35. Tarini, B.A., The current revolution in newborn screening: new technology, old controversies. Arch Pediatr Adolesc Med, 2007. 161(8): p. 767-72.
36. Hewlett, J. and S.E. Waisbren, A review of the psychosocial effects of false-positive results on parents and current communication practices in newborn screening. J Inherit Metab Dis, 2006. 29(5): p. 677-82.
37. DeLuca, J.M., Public Attitudes Toward Expanded Newborn Screening. Journal of pediatric nursing, 2018. 38: p. e19-e23.
38. Joseph, G., et al., Parental Views on Expanded Newborn Screening Using Whole-Genome Sequencing. Pediatrics, 2016. 137(Supplement 1): p. S36-S46.
39. Etchegary, H., et al., Interest in newborn genetic testing: a survey of prospective parents and the general public. Genet Test Mol Biomarkers, 2012. 16(5): p. 353-8.

40. Blom, M., et al., Dilemma of Reporting Incidental Findings in Newborn Screening Programs for SCID: Parents' Perspective on Ataxia Telangiectasia. Front Immunol, 2019. 10: p. 2438.
41. Wiklund, I., et al., New parents' experience of information and sense of security related to postnatal care: A systematic review. Sex Reprod Healthc, 2018. 17: p. 35-42.
42. Skinner, D., et al., Parents' decisions to screen newborns for FMR1 gene expansions in a pilot research project. Pediatrics, 2011. 127(6): p. e1455-e1463.
43. Bailey, D.B., Jr., et al., Design and evaluation of a decision aid for inviting parents to participate in a fragile X newborn screening pilot study. Journal of genetic counseling, 2013. 22(1): p. 108-117.
44. Nicholls, S.G. and K.W. Southern, Parental decision-making and acceptance of newborn bloodspot screening: an exploratory study. PloS one, 2013. 8(11): p. e79441-e79441.
45. Hasegawa, L.E., et al., Parental attitudes toward ethical and social issues surrounding the expansion of newborn screening using new technologies. Public health genomics, 2011. 14(4-5): p. 298-306.
46. Loeber, J.G., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 1. From blood spot to screening result. Journal of inherited metabolic disease, 2012. 35(4): p. 603-611.

SUPPLEMENTARY MATERIAL
SUPPLEMENTAL METHODS

Dutch NBS program

The Dutch NBS program screens for 22 disorders in 2020, performing approximately 170,000 blood spot tests each year. Parents receive information about NBS during pregnancy from the midwife/gynecologist during the first consultation and at 36-42 weeks. After birth, parents will receive additional information when they register their newborn at city hall and from screeners who perform the heel prick (Figure S1). Blood spot collection is primarily performed at home as soon as possible after 72 hours or after 96 hours (if combined with neonatal hearing screening) and no later than 168 hours (Figure S1). At the national level, the NBS program is coordinated and monitored by the National Institute for Public Health and the Environment (RIVM) [1].

TREC analysis

TREC analysis was performed according to the SPOT-itTM kit instructions for use (ImmunoIVD, Stockholm, Sweden) in two screening laboratories (RIVM, Bilthoven and IJsselland Hospital, Capelle aan den IJssel). β-actin served as a control marker. Single 3.2 mm discs (equal to 3.0 µL blood) were punched from heel prick cards into a Filter plate using a Wallac DBS puncher (1296-071, PerkinElmer, Turku, Finland). Samples were rinsed and transferred into an Elution plate for elution at 95˚C. Next, eluted DNA was transferred into a qPCR plate and analyzed in a QuantStudio 5 qPCR system (ThermoFisher, Waltham, Massachusetts, USA). Copy numbers were calculated with the QuantStudio software 1.4.2 (ThermoFisher).

Cut-off value and screening algorithm

From April 2018 to October 2018, newborns with TREC ≤ 6 copies/3.2 mm punch were referred for clinical follow-up, according to the kit instructions (ImmunoIVD). After six months of screening, the TREC cut-off value was increased to ≤ 10 copies/3.2 mm punch from November 2018 to February 2020 to ensure that no atypical SCID cases would be missed. For the complete screening algorithm, see Figure S2.

Diagnostic follow-up protocol

A uniform diagnostic follow-up protocol after abnormal TREC results was established (Figure S3). Clinical evaluation by a pediatrician-immunologist was realized within 72 hours after an abnormal TREC result. Immunophenotyping by flow cytometry included analysis of CD3+ T-cells, CD4+ and CD8+ T-cells, CD56/16 NK-cells and CD19 B-cells and T-cell subsets CD45RA/CD45RO (%) naive T-cells. Newborns with

normal levels of T-cells and without underlying cause for low TREC levels were considered false positive and follow-up was not conducted. In the case of absent or low/abnormal T-cells, initial genetic analysis was performed by whole-exome sequencing and subsequent gene panel analysis of the known causative genes for SCID (Table S1). Newborns with absent T-cells would simultaneously start with the HSCT work-up and while awaiting HSCT, protective measures would be taken. In the case of low or abnormal T-cells, additional immunological diagnostics could be carried out.

Follow-up interviews with parents after an abnormal TREC result

Interviews were conducted with parents after an abnormal SCID screening result to explore what parents experienced during the referral procedure. The semi-structured interview-guide included six topics: 1) views on information about the SONNET-study and information provision during referral, 2) reasons to participate in the SONNET-study, 3) experiences with referral procedure and the follow-up care after an abnormal screening result, 4) trust in the NBS program and 5) current clinical condition of the newborn and 6) psychological wellbeing of the parents. In total, 23 parents were approached for an interview. Parents of newborns who had deceased and parents of newborns who were in the hospital at the time of the referral were not contacted (N = 24). After the interview, parents were asked to complete two questionnaires, the Vulnerable Baby Scale [2] and parental stress OBVL (Opvoedingsbelasting Vragenlijst) questionnaire to assess parental perception of their newborn and themselves. Interviews were either conducted in person or by telephone. The interviews were recorded and transcribed verbatim. Transcripts were coded independently by two researchers using the software MAXQDA 2018 0-5 (VERBI GmbH, Berlin, Germany).

Questionnaire study on parents' perspective on NBS for SCID

To evaluate the perspective of parents on NBS for SCID, a questionnaire was sent to 2000 parents of healthy newborns born in the pilot provinces; N = 400 parents who declined participation in the SONNET-study and N = 1600 parents who participated. Parents were able to send back a printed questionnaire or to fill in the questionnaire online by following a link or scanning a QR-code. Data were collected and analyzed anonymously. Due to privacy reasons, it was not allowed to send reminders. The questionnaire was subdivided into four different sections: 1) opinion on the provided information in the SONNET-study, 2) knowledge about SCID, 3) opinion on NBS for SCID, and 4) demographic information (gender, age, ethnicity, educational level, number of children).

Data analyses

Descriptive statistics were used to summarize the distribution of TREC and β-actin levels in the Dutch newborn pilot population, and the characteristics of the respondents of the questionnaire study. Mann-Whitney U and Kruskal-Wallis tests were used for group comparison. The sociodemographic characteristics of the questionnaire respondents were compared to the Dutch reference population with one sample-t-test for age, chi-square test for trend for number of children and Pearson's chi-square test for other variables. Ordinal variables from scaled items were reported as means. Odds ratios and multivariate logistic regression analyses were used to determine associations between sociodemographic variables and outcome variables such as participation in the SONNET study. By assigning 1 point for each correctly answered knowledge question, an overall knowledge score was created (0-4). P-values < 0.05 were considered statistically significant. Statistical analysis was carried out with SPSS version 25.0 for Windows (SPSS, Inc., Chicago, IL, USA).

SUPPLEMENTAL REFERENCES

1. Blom, M., et al., Introducing Newborn Screening for Severe Combined Immunodeficiency (SCID) in the Dutch Neonatal Screening Program. International Journal of Neonatal Screening, 2018. 4(4): p. 40.
2. Kerruish, N.J., et al., Vulnerable Baby Scale: development and piloting of a questionnaire to measure maternal perceptions of their baby's vulnerability. J Paediatr Child Health, 2005. 41(8): p. 419-23.
3. Statistics Netherlands. Birth; key figures. 2017 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37422eng/table?ts=1564486871296.
4. Statistics Netherlands. Population; key figures. 2018 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37296eng/table?ts=1564487099871.
5. Statistics Netherlands. Households; size, composition, position in the household, 1 January. 2018 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/82905ENG/table?ts=1564487413252.
6. Statistics Netherlands. Labour Force; level of education by personal characteristics. 2019 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/71822eng/table?fromstatweb.
7. RIVM. 2017 2017; Available from: https://draaiboekhielprikscreening.rivm.nl/documenten/stroomschema-uitvoering-hielprikscreening.
8. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. J Allergy Clin Immunol, 2017. 139(3): p. 733-742.

Table S1. SCID gene panel used in the follow-up after an abnormal TREC result

Genes included in SCID gene panel	OMIM gene
ADA	608958
AK2	103020
B2M	109700
CD247	186780
CD3D	186790
CD3E	186830
CD3G	186740
CD8A	186910
CIITA	600005
CORO1A	605000
DCLRE1C	605988
DOCK2	603122
DOCK8	611432
FOXN1 (added per 01-01-2020)	600838
IL2RG	308380
IL7R	146661
JAK3	600173
LAT	602354
LCK	153390
LIG4	601837
NHEJ1	611290
PNP	164050
PRKDC	600899
PTPRC	151460
RAC2	602049
RAG1	179615
RAG2	179616
RFX5	601863
RFXANK	603200
RFXAP	601861
RMRP	157660
STK4	604965
TAP1	170260
TAP2	170261
TAPBP	601962
TTC7A	609332
ZAP70	176947

Table S2. Demographic variables and SCID screening parameters in the Dutch newborn pilot population

Sample size	**N = 140,593**	**TRECs in copies/3.2 mm punch** Median (IQR)	**β-actin in copies/3.2 mm punch** Median (IQR)
All newborns		97 (66-141)	3,564 (2,455-5,079)
Sex			
Male	51.4% (N = 72,209)	92 (62-133)	3,494 (2,409-4,981)
Female	48.6% (N = 68,375)	103 (70-148)	3,641 (2,502-5,194)
	Missing 10	P < 0.01	P < 0.01
Age at sample collection (in hours), median (IQR)	102 (89-121)		
Early collection <72 hours	0.9% (N = 1,181)	86 (59-124)	3,816 (2,633-5,454)
Timely sample collection (72 to 168h)	97.4% (N =136,865)	97 (66-140)	3,564 (2,455-5,077)
Late collection >168 hours	1.7% (N = 2,544)	123 (82-179)	3,503 (2,378-5,048)
	Missing 3	P < 0.01	P < 0.01
Gestational age (in days), median (IQR)	278 (271-285)		
Extremely preterm <32 weeks	1.1% (N = 1,483)	59 (34-95)	3,750 (2,342-5,932)
Preterm (32-36 weeks)	5.4% (N = 7,643)	85 (56-123)	3,374 (2,324-4,835)
Term ≥ 37 weeks	93.5% (N = 131,399)	99 (67-142)	3,576 (2,465-5,085)
	Missing 68	P < 0.01	P < 0.01
Birth weight (in grams), median (IQR)	3450 (3,105-3780)		
Low birth weight <2500 gram	5.5% (N = 7,759)	81 (52-121)	3,460 (2,362-5,093)
Normal birth weight ≥2500 gram	94.5% (N = 132,808)	98 (67-142)	3,571 (2,461-5,078)
	Missing 26	P < 0.01	P < 0.01
Blood transfusion <24 hours sample collection	0.01% (N = 32)	44 (20-77)	3,650 (2,466-5,518)
Yes	99.9% (N = 140,561)	97 (66-141)	3,564 (2,455-5,079)
No			
	Missing 0	P < 0.01	P = 0.596

Notes: TRECs - T-cell receptor excision circles, IQR – interquartile range. Mann-Whitney U test was used for gender, birth weight and <24 hour blood transfusion comparison with TREC/β-actin levels. Kruskal-Wallis test for was used for comparison between age at sample collection and gestational age with TREC/β-actin levels.

Table S3. Diagnoses of 47 infants with (non)-SCID T-cell lymphopenia and false-positive results identified via newborn screening

Classification		Number of newborns (N = 47)	TRECs in copies/ 3.2 mm punch Median (range)
SCID	X-linked SCID (*IL2RG*)	1	0
T-cell impairment syndromes	22q11.2 deletion syndrome (DiGeorge)	4	1.5 (0-8)
	Trisomy 21	2	4.5 (1-10)
	Noonan syndrome	1	0
	Heterozygous *FOXN1* variant	1	6
Secondary T-cell impairment	Multiple congenital anomalies[a]	7	8 (2-13)
	Congenital diaphragmatic hernia	3	7 (2-10)
	Cardiac anomalies	2	8.5 (6-15)
	Gastrointestinal anomalies	2	2 (0-8)
	Chylothorax and hydrops	1	0
	Sepsis/severe infections	6	5.5 (0-18)
	Maternal immunosuppressant us	3	5 (1-16)
	Other neonatal conditions[b]	4	6 (2-9)
Idiopathic T-cell lymphocytopenia		5	5 (2-26)
False-positive		5	5 (0-14)

a. Multiple congenital anomalies included newborns with nemaline rod myopathy (de novo variant *ACTA1*), holoprosencephaly/diaphragmatic hernia due to *GLI1* variant, MADD deficiency and others.

b. Other neonatal conditions included severe asphyxia, dysmaturity, high doses of dexamethasone and start of chemotherapeutics prior to sample collection.

Table S4. Characteristics of the interviewees after an abnormal TREC result (N = 17)

Respondent #	Diagnosis after follow-up	Interview setting	Interview with	Referral via
1	22q11.2 deletion syndrome/DiGeorge	Academic medical center	Mother	General practitioner
2	Lymphopenia due to severe sepsis	Telephone	Father	Hospital
3	Mother used immunosuppressant medication during pregnancy	Home	Mother	General practitioner
4	Mother used immunosuppressant medication during pregnancy	Academic medical center	Both parents	General practitioner
5	Idiopathic T-cell lymphocytopenia	Home	Mother	General practitioner
6	False positive	Home	Mother	General practitioner
7	Idiopathic T-cell lymphocytopenia	Home	Mother	General practitioner
8	Idiopathic T-cell lymphocytopenia	Home	Both parents	General practitioner
9	False positive	Telephone	Mother	General practitioner
10	Idiopathic T-cell lymphocytopenia	Telephone	Mother	General practitioner
11	Mother used immunosuppressant medication during pregnancy	Telephone	Mother	General practitioner
12	False positive	Telephone	Mother	General practitioner
13	False positive	Telephone	Mother	General practitioner
14	22q11.2 deletion syndrome/DiGeorge	Telephone	Mother	General practitioner
15	Noonan syndrome	Telephone	Mother	Hospital
16	Idiopathic T-cell lymphocytopenia	Telephone	Mother	General practitioner
17	False positive	Telephone	Mother	General practitioner

Table S5. Sociodemographic characteristics of the questionnaire respondents (N = 391)

Variables	Respondents questionnaire study N = 391	Reference group Dutch population N (x1000)	P-value
Age in years (range)		Dutch parents[a]	
Mean age mothers	31.8 (20-45)	31.4	P = 0.401
Mean age fathers	35.0 (26-52)	34.2	P = 0.075
Missing	20		
Gender, N (%)		Dutch population age 20-50 years[b]	P <0.01
Female	319 (85.8)	3,266 (49.7)	
Male	53 (14.2)	3,304 (50.3)	
Missing	19		
Background, N (%)		Dutch population age 20-50 years[c]	P <0.01
Dutch	312 (83.9)	4,675 (70.6)	
Other	60 (16.1)	1,932 (29.4)	
Missing	19		
Civil registry, N (%)		Dutch population age 20-50 years[d]	P <0.01
Living together/Married	362 (97.3)	2,024 (78.4)	
Single	10 (2.7)	572 (21.6)	
Missing	19		
Highest level of education, N (%)		Dutch population age 20-50 years[e]	P <0.01
Low	18 (4.9)	585 (30.9)	
Middle	101 (27.2)	1,643 (38.1)	
High	252 (67.9)	1,908 (29.4)	
Missing	20		
Number of children, N (%)		Dutch parents[f]	P = 0.17
1	181 (48.7)	71.9 (44.2)	
2	129 (34.7)	62.5 (38.5)	
≥3	62 (16.6)	28.1 (17.3)	
Missing	19		

Missing values were excluded from the percentages.

a. Reference population Dutch Parents [3]. One sample T-test.
b. Reference population Dutch population age 20-50 years [4]. χ^2 test
c. Background was coded as 'Dutch' if both parents were born in the Netherlands. Reference population Dutch population age 20-50 years [4]. χ^2 test
d. Reference population Dutch population households [5]. χ^2 test
e. Low: primary education, lower vocational education, lower and middle general secondary education. Middle: middle vocational education, higher secondary education, and pre-university education. High: higher vocational education and university. Reference population Dutch population age 25-45 years [6]. χ^2 test
f. Reference population Dutch parents [5]. χ^2 test for trend.

Table S6. Participation in NBS, health status of the children and familial disorders of the questionnaire respondents (N = 391)

	Respondents questionnaire study N (%)
Did all your children participate in the Dutch NBS program?	
Yes	364 (96.6)
No	5 (1.4)
Missing	22
What was the NBS result for your child(-ren)?	
Normal	364 (99.4)
Abnormal	1 (0.3)
I would rather not say	1 (0.3)
Missing	25
Are your children healthy? [a]	
Yes	362 (97.3)
No	8 (2.2)
I would rather not say	2 (0.5)
Missing	19
Do you have a family member with a hereditary disorder? [b]	
Yes	51 (13.7)
No	292 (78.7)
I do not know	26 (7.0)
I would rather not say	2 (0.6)
Missing	20

Missing values were excluded from the percentages.

a. Answers included a wide variety of hereditary disorders including Down Syndrome, trisomy 18, asthma, neurological and congenital anomalies.

b. Answers included a broad spectrum of disorders such as malignancies, metabolic diseases, diabetes mellitus, cardiovascular diseases, inflammatory bowel disease and autoimmune disorders.

Table S7. Knowledge questions about SCID and the percentage of parents answering them correctly based on participation in the SONNET study (N = 348)

	Knowledge questions	**Participated in the SONNET-study N = 332 % (N) that answered correctly:**	**Declined participation in the SONNET-study N = 16 % (N) that answered correctly:**	**P-value***
1.	In the Netherlands, approximately 200 children are born with SCID each year. *False*	75.4% (248)	75.0% (12)	0.568
2.	Children with SCID get severe infections during the first months after birth. *True*	93.3% (307)	93.8% (15)	0.711
3.	The treatment for SCID is stem cell transplantation. *True*	92.7% (305)	75.0% (12)	0.032
4.	SCID can be cured if detected in an early phase. *True*	90.6% (298)	81.3% (13)	0.201

Missing values (N = 10) and parents who could not remember whether they participated (N = 33) are excluded from the calculations. *Fisher exact test

Table S8. Parental support of scientific research and NBS for SCID (N = 377)

Questionnaire statement	Degree of support[a] (%)			Rating mean (SD)
	(Fully) disagree	Neutral	(Fully) agree	
Scientific research is required to prevent diseases	2.6	4.3	93.1	4.6 (0.75)
Scientific research is required to improve treatment of diseases	2.4	2.4	95.2	4.6 (0.72)
SCID is a severe disorder and I want this disorder to be detected in my child as early as possible	3.7	13.1	83.1	4.3 (0.90)
I think it is important that SCID is included in the newborn screening program	2.1	17.6	80.2	4.2 (0.82)
I want as much information as possible about my child's health	7.7	15.7	76.5	4.0 (0.96)
I want to be reassured that my child does not have SCID	9.9	20.3	69.9	3.9 (1.05)
I do not worry about the health of my child	23.0	22.0	54.9	3.4 (1.19)
My family/ partner wanted the SCID test to be performed for my child	36.0	39.8	24.2	2.7 (1.23)
I only want my child tested for SCID once the study has been completed and SCID has been included in the newborn screening program	69.6	14.8	15.7	2.2 (1.13)
I think I have a high risk of getting a child with SCID	71.7	27.4	0.8	1.8 (0.87)
I do not want to participate in scientific research	85.8	9.1	5.0	1.6 (0.90)
The person who performed the heel prick advised me to participate in SCID screening	85.1	13.0	1.9	1.5 (0.81)

SD = Standard deviation. [a] Five-point rating scale: 1 = fully disagree; 5 = fully agree converted into three-point scale. Missing values are excluded from the percentages.

Table S9. Variables and the likelihood of participation in pilot study: multivariate logistic regression analysis

	Participation in pilot study for NBS SCID (yes)		
Predictor variable	**B**	**SE**	**P-value**
Age	-0.031	0.059	0.593
Gender (female)	0.626	0.800	0.434
Ethnicity (Dutch)	0.797	1.087	0.464
Civil registry (Living together/married)	0.824	1.502	0.584
Educational level (high)	0.989	0.611	0.105
Number of children	**-0.593**	**0.294**	**0.044**
Having a child with a disorder (yes)	0.138	1.555	0.930
Having a family member with a hereditary disease (yes)	0.341	0.824	0.679
Received information via screener/midwife (yes)	-0.113	0.591	0.848
Read leaflet (yes)	-0.740	0.585	0.206
Knowledge score	0.625	0.335	0.062

Multivariate logistic regression analysis (N = 269 valid cases included for analysis) with standardized regression coefficients β and standard error (SE). In **bold** the significant predictor variable for participation in the SCID pilot study.

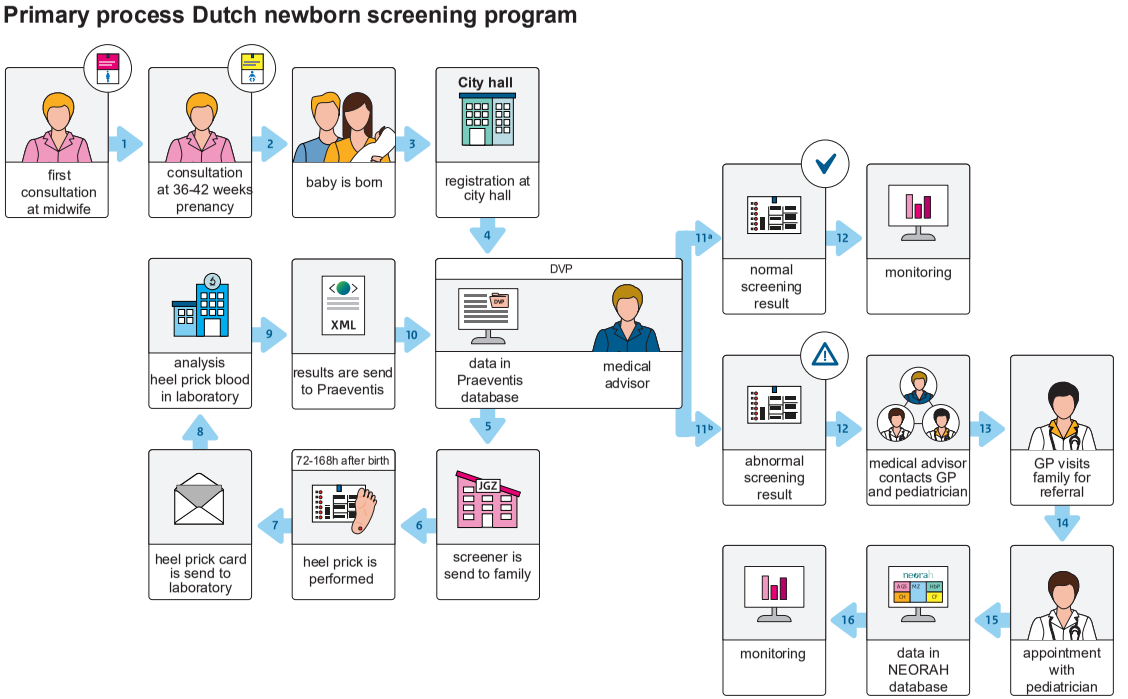

Figure S1. Primary process Dutch newborn screening program. Figure available via RIVM-website [7]. Expecting parents will receive information about the NBS program during the first and second consultation with the midwife (1-2). During registration at city hall, an information brochure will be handed out as well (3). The screening organizations (JGZ) will be informed about the registration of the newborn, after which screeners will visit the family to perform the heel prick (6). The heel prick card is send by post to one of the five screening laboratories (7). The heel prick cards are then analyzed and the results are registered in the national monitoring database Praeventis (8-9). Abnormal results are forwarded to the general practitioner (GP) and pediatrician by the medical advisor (12-13). Medical advisors coordinate logistics of the referral procedure. GPs will visit the family to inform them about the referral after which the family will visit the pediatrician for follow-up diagnostics (14).

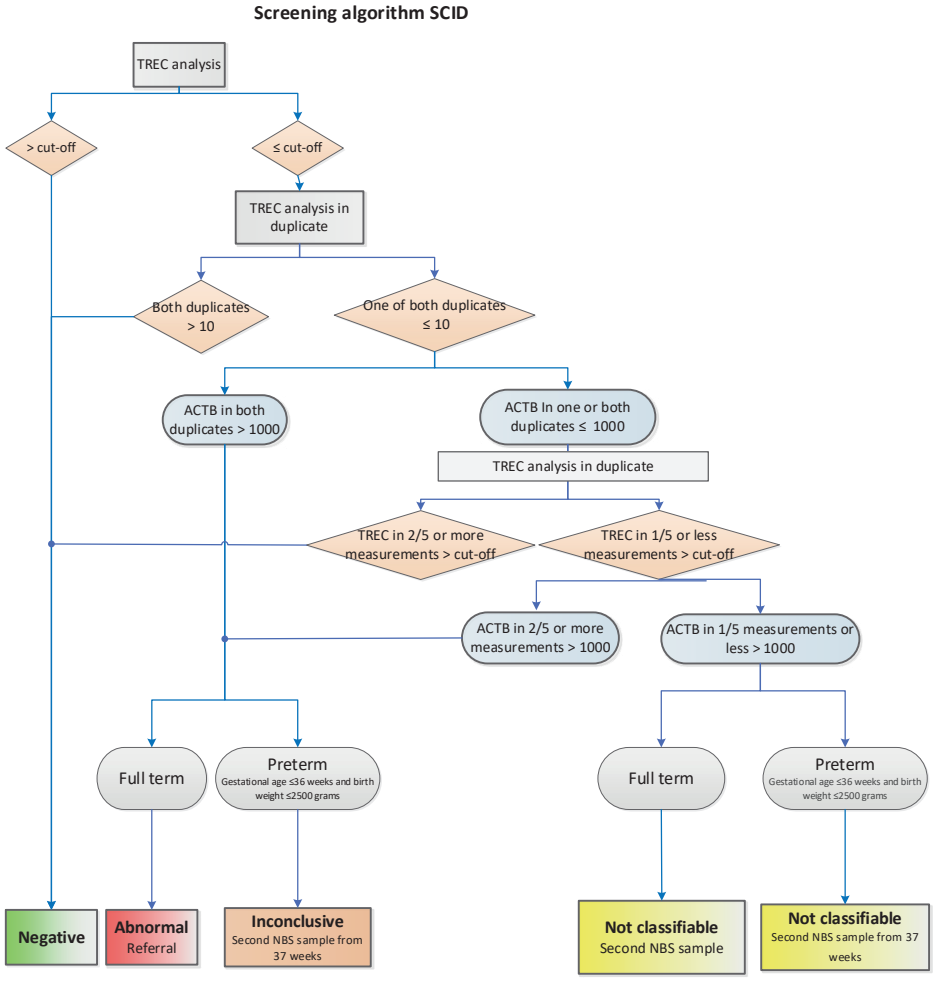

Figure S2. SCID screening algorithm. Samples with low TREC levels require repeated analysis in duplicate. Full term infants with low TREC levels were referred to an academic medical center for follow-up diagnostics. Preterm infants with abnormal results required a second specimen to be collected from the corrected age of 37 weeks.

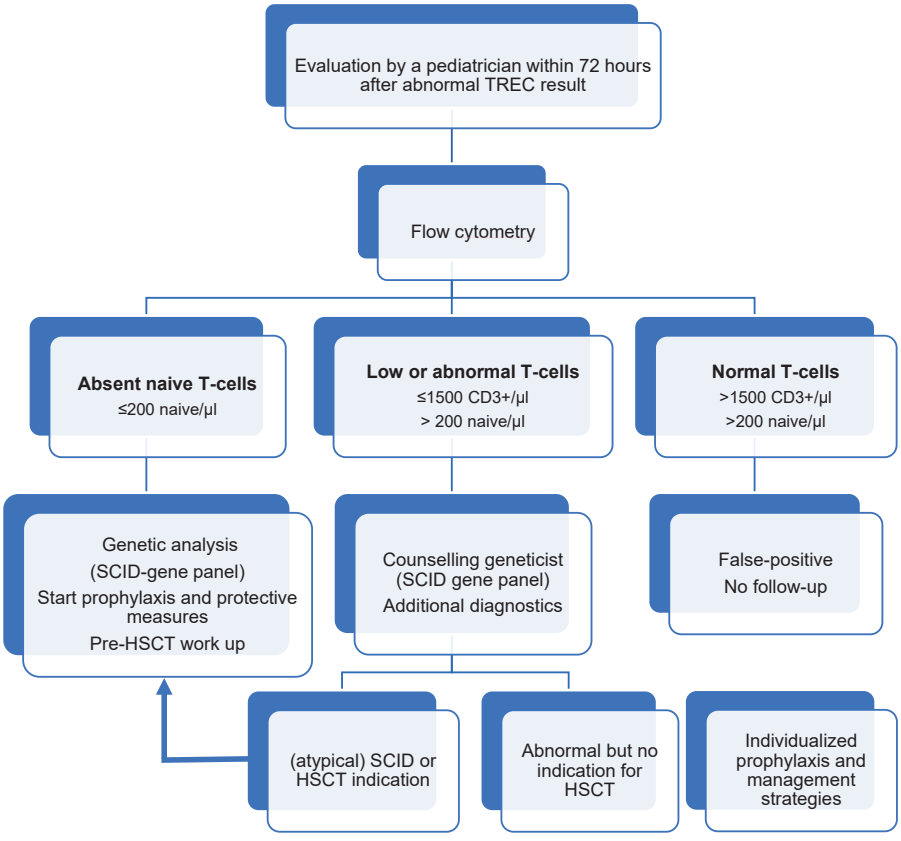

Figure S3. Uniform follow-up protocol used after an abnormal SCID screening result. Absent T-cells were defined as ≤ 200 naive CD4+ T-cells/µl blood. Low or abnormal T-cells were defined as ≤ 1500 CD3+ positive T-cells/µl blood > 200 CD4+ naive T-cells/µl blood. Normal T-cells were defined as > 1500 CD3+ positive T-cells/µl blood > 200 CD4 naive T-cells/µl blood (in accordance with Dorsey *et al*. 2017 [8].

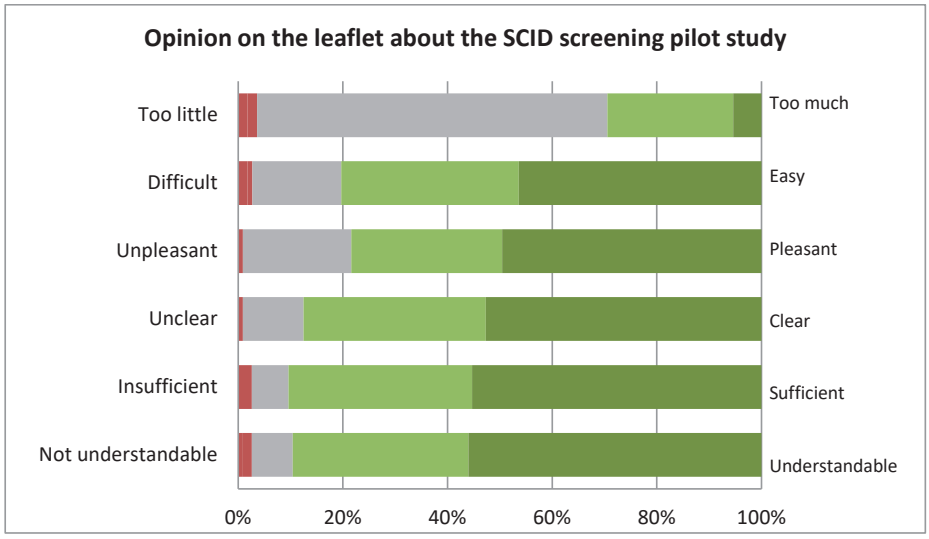

Figure S4. Opinion of parents on the leaflet about the SCID screening pilot study (N = 118). Only parents who answered that they read the leaflet were routed to this question. The red bars represent the percentage of parents who (totally) agreed with the words on the left side of the figure. The gray bars represent the percentage of participants with a neutral opinion towards the word pairs. The green bars represent the percentage of parents who (totally) agreed with the words on the right side of the figure.

CHAPTER 5

Second tier testing to reduce the number of non-actionable secondary findings and false-positive referrals in newborn screening for severe combined immunodeficiency

Maartje Blom, Ingrid-Pico Knijnenburg, Sandra Imholz, Lotte Vissers, Janika Schulze, Jeannette Werner, Robbert Bredius and Mirjam van der Burg

Journal of Clinical Immunology, 2021; 41 (8): 1762-1773

ABSTRACT

Purpose
Newborn screening (NBS) for severe combined immunodeficiency (SCID) is based on the detection of T-cell receptor excision circles (TRECs). TRECs are a sensitive biomarker for T-cell lymphopenia, but not specific for SCID. This creates a palette of secondary findings associated with low T-cells that require follow-up and treatment or are non-actionable. The high rate of (non-actionable) secondary findings and false-positive referrals raises questions about the harm-benefit-ratio of SCID screening, as referrals are associated with high emotional impact and anxiety for parents.

Methods
An alternative quantitative TREC PCR with different primers was performed on NBS cards of referred newborns (N = 56) and epigenetic immune cell counting was used as for relative quantification of CD3+ T-cells (N = 59). Retrospective data was used to determine the reduction in referrals with a lower TREC cut-off value or an adjusted screening algorithm.

Results
When analyzed with a second PCR with different primers, 45% of the referrals (25/56) had TREC levels above cut-off, including four false-positive cases in which two SNPs were identified. With epigenetic qPCR, 41% (24/59) of the referrals were within the range of the relative CD3+ T-cell counts of the healthy controls. Lowering the TREC cut-off value or adjusting the screening algorithm led to lower referral rates but did not prevent all false-positive referrals.

Conclusions
Second tier tests and adjustments of cut-off values or screening algorithms all have the potential to reduce the number of non-actionable secondary findings in NBS for SCID, although second tier tests are more effective in preventing false-positive referrals.

INTRODUCTION

Newborn screening (NBS) for severe combined immunodeficiency (SCID), the most profound form of inborn errors of immunity (IEI), improves outcomes for patients by preventing severe infections and early death. SCID is characterized by severe T-cell lymphopenia that is variably associated with an abnormal development of B- and/or natural killer (NK)-cells [1]. Patients with SCID are usually born asymptomatic but develop life-threatening infections in the first months of life [2]. Early detection by NBS enables prompt immune-restoring therapy such as hematopoietic stem cell transplantation (HSCT) or in selected cases gene therapy before infections have occurred [3-5]. An increasing number of countries are adopting the T-cell receptor excision circle (TREC) assay into their screening programs to identify newborns with SCID in a pre-symptomatic phase [6-11]. TRECs are circular excised fragments of DNA formed during the T-cell receptor gene rearrangement. The δRec-ϕJα TREC is formed as a byproduct in approximately 70% of developing T-lymphocytes that express αβ and can therefore serve as a marker for thymic output [6]. The number of TREC copies is an indicator of thymic production of naïve T-cells as TRECs are stable and do not replicate during mitosis. TRECs can be detected in dried blood spots (DBS) by quantitative amplification using primers flanking across the joint of the circle [6, 12]. Absent or low levels of TRECs indicate reduced levels of newly formed T-lymphocytes regardless of the underlying cause.

TRECs are a highly sensitive biomarker for T-cell lymphopenia, but a non-specific marker for the primary target disease SCID, introducing the field of NBS to a palette of neonatal conditions and disorders associated with low T-cells around birth [13]. Low or absent TRECs can be identified in newborns with T-cell impairment syndromes such as Down syndrome, DiGeorge syndrome or ataxia telangiectasia. In addition, newborns with T-cell impairment secondary to other neonatal conditions such as cardiac/gastrointestinal anomalies, chylothorax/hydrops or maternal immunosuppressant use, patients with idiopathic T-cell lymphopenia or preterm children can have low TREC levels at birth. Finally, NBS for SCID can result in false-positive referrals; newborns with normal number of naïve T-cells as determined by flow cytometric analysis and no clinical explanation for the low TREC levels [14, 15]. The diagnosis of SCID can only be made after follow-up diagnostics including immunophenotyping and confirmatory genetic testing.

All countries that have implemented NBS for SCID are struggling with a low positive predictive value of SCID screening; the number of identified SCID patients is relatively low compared to the high number of other disorders with low TREC levels (secondary findings) [7, 10, 11, 14, 16-18]. This high number of secondary findings is met with hesitations

by policy makers involved in implementation of SCID in NBS programs. A distinction can be made between actionable secondary findings where treatment or prevention achieves substantial health gain for the child and non-actionable secondary findings that may be relevant prognostically, but for which no treatment options are available or treatment options have no significant impact on outcomes [19]. Reporting actionable T-cell lymphopenia in which children will benefit from antibiotic prophylaxis, endorsing protective measures or by not receiving life-attenuated vaccines is undisputed in the field of neonatal screening [20, 21]. However, non-actionable secondary findings and false-positive referrals raise questions about the harm-benefit ratio of screening and NBS programs should make an effort to prevent referral of these cases. High referral rates can be associated with a high work load for downstream referral centers and high diagnostic costs. More importantly, referral procedures are associated with high emotional impact for parents [17]. Even parents with a confirmed healthy newborn after follow-up can perceive their newborn as more 'vulnerable' implying some effect of the referral procedure with the associated feelings of anxiety [22, 23]. There is an urgent need to reduce the number of non-actionable secondary findings and false-positive referrals in NBS for SCID for all countries that have implemented SCID screening.

Several NBS programs have tried to find the appropriate screening strategy that balances a high sensitivity, avoiding missing SCID patients, while preventing high referral rates (ranging from 0.01% to 0.09%) [7, 10, 11, 14, 16-18]. Lowering cut-off values, requesting second NBS cards or adjusting screening algorithms for preterms could reduce the number of referrals without the need of introducing a second tier test [11, 14, 24-30]. Other programs have chosen to include a second tier test after initial TREC analysis such as next generation sequencing (NGS) with gene panels [31, 32]. This study explores other options of second tier testing after TREC analysis such as PCR with different TREC primers and epigenetic immune cell counting in order to reduce the number of non-actionable secondary findings and false-positives. Performing a PCR with primers at different positions as a second tier could prevent false-positive referrals caused by TREC region variations leading to primer/probe annealing problems [33]. Epigenetic immune cell counting as a second tier allows the measurement of relative (epi) $CD3^+$ T-cells counts serving as more direct marker for absolute T-cells [34]. Finally, retrospective data will be used to determine whether the number of (non-actionable) secondary findings and false-positives could have been reduced with a lower cut-off value or an adjusted screening algorithm. By exploring these different options, this study will make an effort to reduce the number of non-actionable secondary findings and false-positive referrals that are associated with NBS for SCID.

METHODS

Study population

The SONNET-study (Dutch implementation pilot) screened 201,470 newborns for SCID from April 2018 to December 2020 [17]. NBS cards of these newborns were included in this study. The SONNET-study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

NBS cards of newborns with low TRECs (N = 56) and anonymized healthy controls (N = 80) were analyzed with a second PCR with different TREC primers. Epigenetic immune cell counting was performed on NBS cards of anonymized healthy controls (N = 331) and newborns with low TRECs (N = 59). DNA was isolated for TREC region sequencing from NBS cards of healthy controls (N = 12), idiopathic T-cell lymphopenia cases (N = 4) and false-positive referrals (N = 8). Some NBS cards of referred newborns were excluded for second tier analysis due to insufficient DBS material or parental objection to anonymized scientific research with NBS cards. The use of NBS cards was approved by the National Institute for Public Health and the Environment (RIVM; no 2019-3).

TREC measurements

Initial TREC measurements were performed with the SPOT-itTM Neonatal Screening kit (ImmunoIVD, Stockholm, Sweden) according to manufacturer's instructions and a preset screening algorithm [17]. As a second tier option, TREC levels were measured with the NeoMDx TREC/KREC/SMN1 multiplex assay (PerkinElmer, Turku, Finland) according to the manufacturer's instructions. RRP30 was used as internal control. NBS samples were punched in a 96-wells plate after which wash solution and elution solution were added in turn before different incubation steps. After DNA extraction, 3 µL of DNA was added to 12 µL of master-mix. The PCR plate was sealed and analyzed on a QuantStudio 5 qPCR system.

Epigenetic immune cell counting

Epigenetic immune cell counting was performed by amplification of cell type-specific demethylated genomic regions according to the protocol of the manufacturer (Epimune GmbH, Berlin, Germany). In short, DNA was extracted from three 3.2 mm blood punches by adding 68 µL lysis buffer, 11 µL of proteinase K followed by lysis at 56 °C for 15 minutes with 900 rpm shaking (ThermoMixer C, Eppendorf, Hamburg, Germany). Ammonium bisulfite (180 µl) and tetrahydrofurfuryl alcohol (TFHA; 60 µl) were added followed by incubation for 45 minutes at 80 °C, after which binding buffer (580 µl) and isopropanol (380 µl) were added. Punches were removed by transferring the mixture into a fresh 2-ml tube and magnetic beads (MagBind Particles HDQ) were

added for DNA binding. After two extensive washing steps and a drying step at 65 ˚C for 10-15 minutes without shaking, 40 µL of elution buffer was added. The samples were incubated at 65 ˚C for 7-10 minutes at 1400 rpm after which the eluate was transferred into fresh 0.2 ml tubes. Converted DNA was stored at -20 ˚C. For qPCR, 1.5 µl of the DNA was pipetted into a 384 wells plate in triplicate, followed by 3.5 µl of the CD3+ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primer/probe master-mix. The plate was sealed and analyzed using the QuantStudio 6 Flex qPCR system (ThermoFisher, Waltham, Massachusetts, USA). Relative (epi) CD3+ T-cell counts (% of CD3 demethylated copies of GAPDH demethylated copies) were calculated as previously described [34].

TREC sequencing

Based on the primer/probe flanking regions provided by the manufacturer (ImmunoIVD), new primers up- and downstream of the flanking regions were designed (TREC forward: [GGCAAAATGGGGCTCCTG]; TREC reverse [GACATTTGCTCCGTGGTCTG]). DNA was extracted from three 3.2 mm punches using the GenElute Mammalian Genomic DNA Miniprep kit (SIGMA-Aldrich, Saint Louis, Missouri, USA) with an adapted protocol. Isolated DNA was mixed with 2.5 µL 10x Gold buffer, 1.5 µL 25mM MgCl, 0.25 uL 20mM dNTP's, 0.5 µL 10mM primers and 0.1 µL polymerase Ampli TagGOLD (5U/uL). PCR amplification was done by a hot start, followed by 40 cycles of 30" 94˚C denaturation step, 45" 63 ˚C primer annealing and 1'30" 72 ˚C elongation step, finished with 10' 72 ˚C. Sanger sequencing was facilitated by the Leiden Genome Technology Center (LGTC).

Adjustment of the TREC cut-off value and screening algorithm

Based on retrospective data of the SONNET-study [17], the number of secondary findings and false-positive referrals were determined with a different cut-off value for the initial TREC measurements (TREC ≤ 6 copies/punch instead of TREC ≤10 copies/punch with SPOT-it assay) and with an adjusted screening algorithm. As part of the follow-up plan, peripheral blood of referred newborns used for flow cytometry (7 to 10 days after birth) was spotted on filter paper and reanalyzed with the SPOT-it TREC assay. Based on this data, it was determined which newborns would be directly referred (urgent TREC positive cases with TRECs 0-2 copies/punch) and which newborns would have been referred after a second NBS card (newborns with TRECs 2-10 copies/punch) with a new screening algorithm.

Statistical analysis

Descriptive statistics were used to summarize the distribution of TREC levels and relative (epi) CD3+ T-cell counts. For correlation analysis, Pearson r correlation tests were used, while unpaired t-tests were used for group comparison. Epigenetic CD3/

GAPDH copies were log-transformed and used to estimate a normal distribution with 99.9% confidence interval. P-values < 0.05 were considered statistically significant. All P-values are two-sided. Statistical analysis was carried out with SPSS version 25.0 for Windows (SPSS, Inc., Chicago, IL, USA).

RESULTS

Results SONNET-study

In total, 62 out of 201,470 newborns were identified in the SONNET-study with low TREC levels (April 2018 to December 2020). These newborns were referred for follow-up diagnostics, leading to a referral rate of 0.03%. One X-linked SCID patient was identified with absent TRECs and absent T-cells [17]. In the other 61 newborns, eight newborns had normal flow cytometry results without a known underlying cause for the low TREC levels (false-positives). There were 53 newborns with non-SCID T-cell lymphopenia of which the diagnoses are specified in Table S1.

SNPs identified in the TREC region of false-positive referrals

Eight of 62 (13%) referred newborns had normal flow cytometric results and no clinical explanation for the low TREC levels. As part of the follow-up plan, peripheral blood used for flow cytometry was spotted on filter paper and reanalyzed with the SPOT-it TREC assay for seven false-positive cases. Three false-positive cases had normal TREC levels in the peripheral blood DBS, as would be expected with normal absolute T-cell counts measured in the same blood sample (data not shown). However, four false-positive cases with normal immunophenotyping had low or undetectable TRECs in repeated TREC analysis on peripheral blood DBS (Table 1). Variations in the TREC region might lead to primer/probe annealing problems and therefore amplification failure in these false-positive referrals. With sequencing, SNPs were identified in the TREC region (defined by manufacturer, personal communication) of these four false-positive cases, whereas no variations were found in the false-positive cases with normalized TREC levels in peripheral blood (Figure 1, Table 1). Case 1 had two heterozygous SNPs, whereas Case 2 had one heterozygous SNP. Case 3 and 4 had complete amplification failure of TREC and had a SNP present on both alleles (Figure 1). The presence of a homozygous SNP might lead to a complete failure of TREC amplification, whereas the presence of two heterozygous SNP will probably lead to less efficient amplification, but not in absence of TRECs. No variations were found in healthy control neonates (N = 12) and referred newborns with idiopathic T-cell lymphopenia (N = 4).

Table 1. SNPs identified in primer/probe binding sites of false-positive referrals with low/absent TREC levels.

	TREC from initial NBS cards in triplicate (copies/punch)	TREC from peripheral blood card in triplicate (copies/punch)	SNP rs377686467[a]	SNP rs1466932014[b]
Case 1	2 – 3 – 6	7 – 3 – 7	G/T	G/A
Case 2	5 – 3 – 3	11 – 10 – 5	G/T	
Case 3	0 – 0 – 0	1 – 0 – 0	T/T	
Case 4	0 – 0 – 0	0 – 0 – 0	T/T	
Case 5	10 – 14 – 6	80 – 114 – 96	No SNP identified	
Case 6	5 – 11 – 10	114 – 41 – 127	No SNP identified	
Case 7	5 –13 – 8	72 – 66 – 102	No SNP identified	
Case 8	1 – 3 – 1	Not measured	No SNP identified	

a dbSNP: NC_000014.9:g.22475276G>T; allele frequency of T=0.0005 (1/2188, ALFA Project) [43].

b dbSNP:NC_000014.9:g.22386840G>A; allele frequency of A= 0.00008 (1/11862, ALFA Project) [44].

PCR with different primers as second tier test

Because of the presence of SNPs in the TREC region, the commercially available NeoMDx PCR with primers at other positions was used as a second tier. Referred newborns with low TREC levels (N = 56), including the eight false-positives, and healthy newborns (N = 80) were analyzed. Mean TREC value of healthy controls was 2844 copies/10^5 cells (range 152 - 6522 copies/10^5 cells), whereas the mean TREC value of the referrals (N = 56) was 387 copies/10^5 cells (range 0 - 4348 copies/10^5 cells). Pearson *r* correlation between TREC levels measured with the SPOT-it kit assay and the NeoMDx assay was 0.74 ($P < 0.001$). Of the referred newborns, 45% of the referrals (25 out of 56) had TREC levels above the cut-off value proposed by the manufacturer (≤ 242 copies/10^5 cells) (Figure 2). Diagnoses below cut-off included SCID (N = 1), syndromes with T-cell impairment (N = 6), idiopathic T-cell lymphopenia (N = 3) and secondary T-cells impairment due to various reasons (N = 21) (Table S1 and Table S2). All false-positive cases had TREC levels above cut-off and in the range of the healthy controls (range 602 - 4348 copies/10^5 cells). One newborn with TRECs of 19 copies/punch measured with the SPOT-it assay had TREC levels below cut-off measured with the NeoMDx assay (TREC 153 copies/10^5 cells).

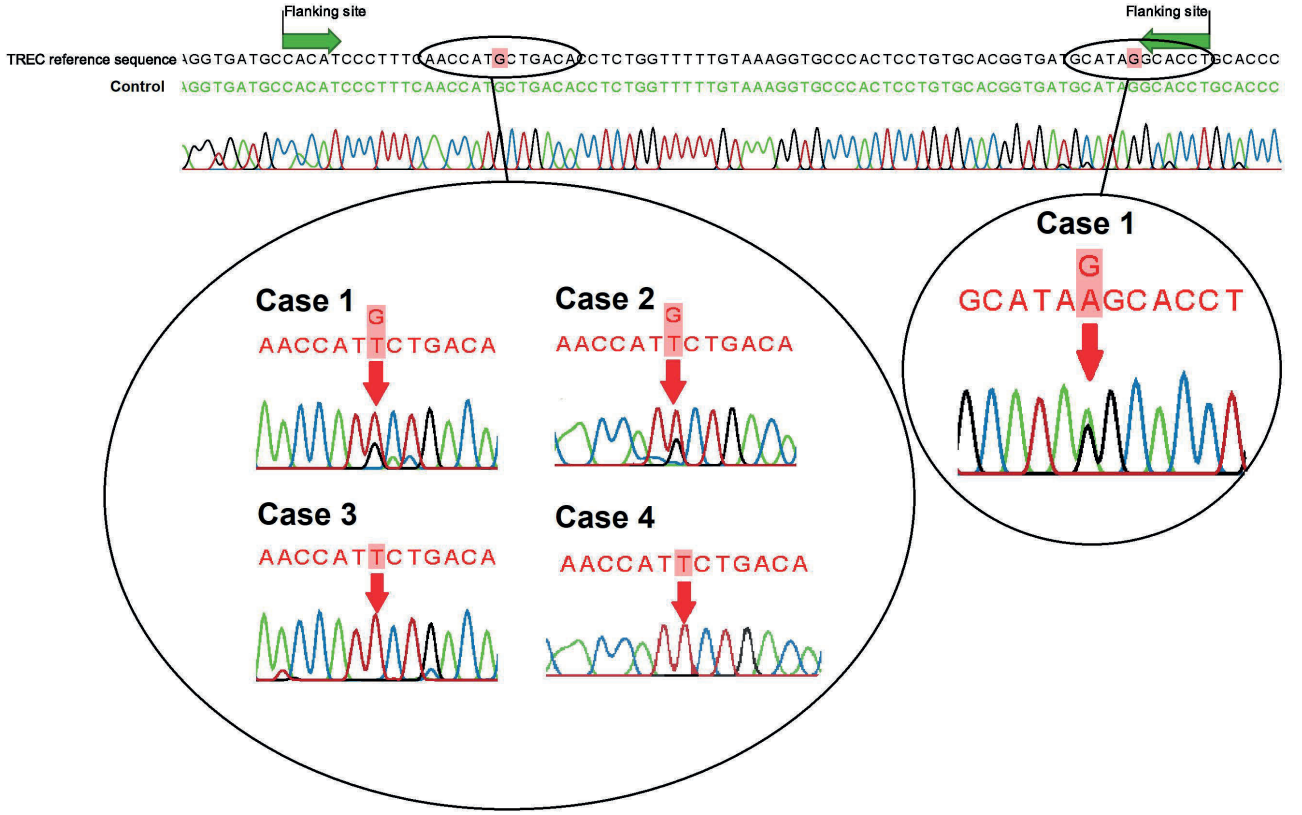

Figure 1. Sequence analysis of TREC region of false-positive referrals with normal flow cytometry and low/undetectable TREC levels in peripheral blood used for flow cytometry spotted on filter paper (N = 4). The flanking regions provided by the manufacturer are depicted with green arrows. SNPs (G>T and G>A) are indicated with the red arrows.

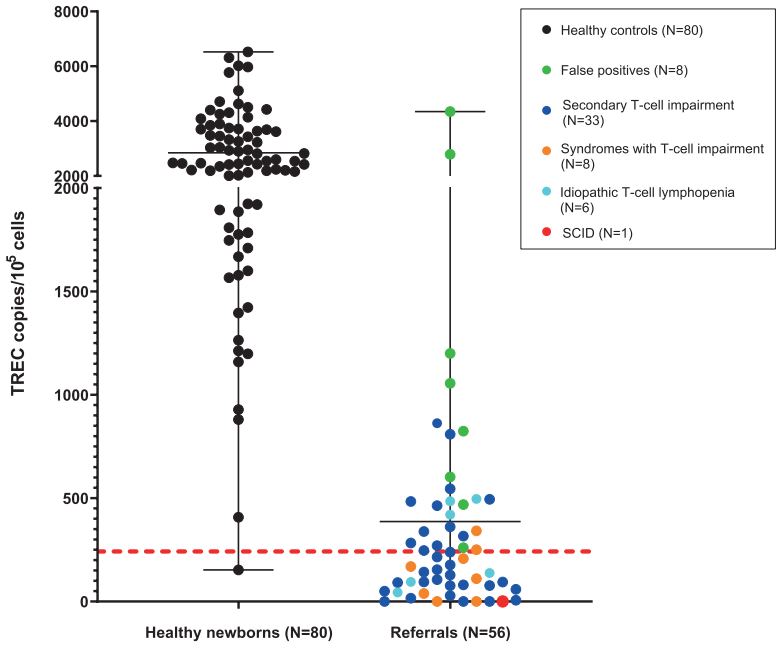

Figure 2. TREC levels in copies/10^5 cells of healthy newborns (N = 80) and referred newborns (N = 56) measured with the NeoMDx assay (PerkinElmer). The red dotted line is the cut-off of the manufacturer at TREC ≤ 242 copies/10^5 cells. The black line shows the mean and range of the TREC copies/10^5 cells. Diagnoses of referrals are categorized as SCID (red), false-positive cases (green), idiopathic T-cell lymphopenia (turquoise), syndromes with T-cell impairment (orange) and secondary T-cell impairment (blue).

Epigenetic immune cell counting as a second tier test

Next, epigenetic immune cell counting was studied as second tier test. This assay is based on amplification of a T-cell-specific demethylated genomic region and measurement of relative (epi) CD3+ T-cell counts in DBS. Mean relative (epi) CD3+ T-cell count as a percentage of leukocytes (CD3%) in healthy newborns was 33.7% (N = 331; range 11.85 – 75.47%), while mean relative (epi) CD3+ T-cell count for referred newborns with low TRECs was 11.6% (N = 59; range 0.09 - 52.60%) ($P < 0.001$). Pearson *r* correlation between TRECs and unmethylated CD3 copies was 0.59 ($P < 0.001$), suggesting a moderate correlation, which implies that epigenetic qPCR can generate different results as a second tier compared to TREC analysis as a first tier. Twenty-four of 59 referrals had relative (epi) CD3+ T-cell counts in the range of healthy controls (41%), including all false-positive cases with confirmed SNPs (Figure 3). For (epi) CD3+ T-cells and GAPDH measurements, 15 out of 59 referrals with low TREC levels (25%) fell within the 99.9% confidence interval ellipse of the healthy controls (Figure 4). The (non)-

actionable diagnoses of these potentially prevented referrals are listed in Table S1 and Table S2. Pearson *r* correlation between absolute CD3+ T-cell numbers determined with flow cytometry and TREC levels measured in peripheral blood of 36 referred newborns was 0.57 (P < 0.001). In contrast, a strong correlation was found (*r* = 0.86 (P < 0.001)) between absolute T-cell numbers and relative (epi) CD3+ T-cell counts as a percentage of total leukocytes measured with epigenetic qPCR.

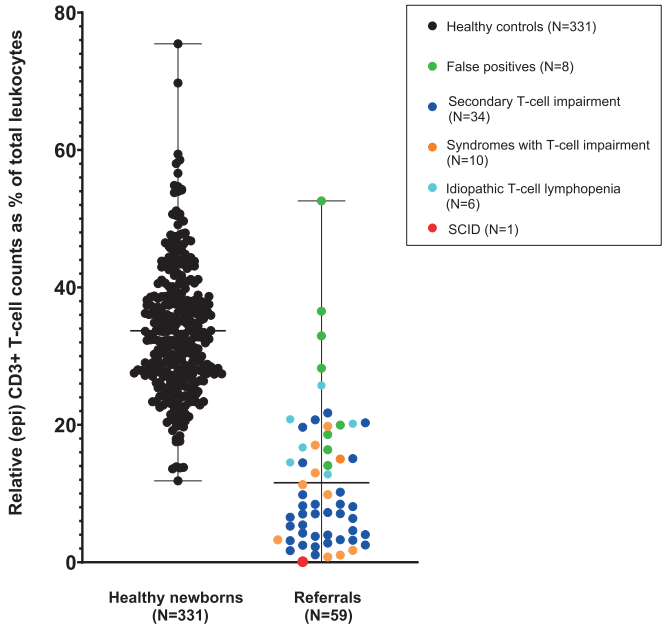

Figure 3. Relative (epi) CD3+ T-cell counts as a percentage of total leukocytes of healthy newborns (N = 331) and referred newborns (N = 59) measured with epigenetic qPCR (Epimune GmbH). The mean and range are depicted with a black line. Diagnoses of referrals are categorized in SCID (red), false-positive cases (green), idiopathic T-cell lymphopenia (turquoise), syndromes with T-cell impairment (orange) and secondary T-cell impairment (blue).

Effect of adjustment of the TREC cut-off value on number of referrals

When applying a lower TREC cut-off value of ≤ 6 copies/punch instead of a cut-off value of TREC ≤ 10 copies/punch to the retrospective data of the referrals in the SONNET-study, 37 of 62 referrals (60%) had TREC levels below 6 copies/punch (Table S1). As a variation in the TREC region of the false-positive referrals can lead to TREC amplification failure and very low or absent TREC levels, lowering the cut-off would not prevent this type of referrals.

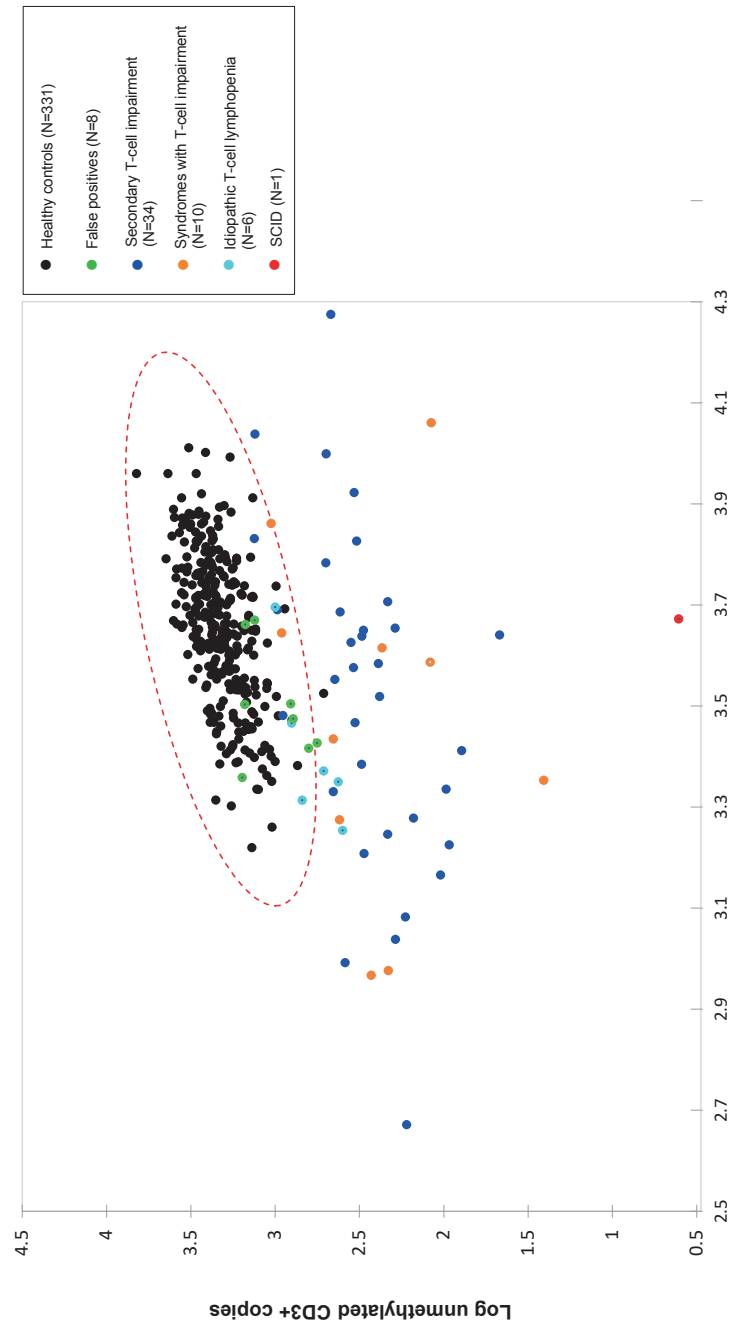

Figure 4. Log-transformed epigenetic CD3/GAPDH copies with a 99.9% confidence interval (red ellipse). Healthy newborns (N = 331) are depicted in black. Diagnoses of referrals are categorized in SCID (red), false-positive cases (green), idiopathic T-cell lymphopenia (turquoise), syndromes with T-cell impairment (orange) and secondary T-cell impairment (blue). 25% (15 out of 59) of the referrals with low TREC levels fell within the 99.9% confidence ellipse of the healthy controls.

Effect of adjustment of the screening algorithm on number of referrals

In the SONNET study all children with TRECs below the cut-off of 10 were referred (N = 62). However, if the screening algorithm would be adjusted in such a way that only newborns with TRECs between 0 - 2 copies/punch were referred directly and newborns with TRECs between 2 - 10 copies/punch would require a second confirmation NBS card, only 14 out of 62 (23%) would have been directly referred; 48/62 (77%) would require a second NBS card. Based on retrospective analysis of DBS of peripheral blood taken on the day of the presumed confirmatory NBS card, 17 out of 32 tested newborns (53%) had a normal TREC levels (above cut-off) and would not have been referred according to this adjusted screening algorithm. Mainly newborns with false-positive screening results without a SNP and newborns with secondary T-cell impairment that resolved in the first week after birth had normal TREC levels in peripheral blood (Table S1).

Effect of combined strategies and second tier tests on number of referrals

Combining data of the different screening strategies and second tier tests show both overlap and differences between the identified and not identified actionable and non-actionable secondary findings (Figure 5). As previously mentioned, not all referrals were analyzed with all screening strategies due to insufficient DBS material or parental objection to anonymized scientific research with NBS cards (Figure 5A/5C, Table S1 and Table S2). Only referrals analyzed with all four screening strategies and second tier test were included for overlap analysis (N = 42 out of 62 referrals) (Figure 5B/5D). All screening strategies, second tier tests and combinations of both are able to identify patients with SCID (N = 1), heterozygous FOXN1 variant (N = 1), Noonan Syndrome (N = 1), RECQL4 (N = 1), while not identifying or 'missing' one 22q11.2 deletion syndrome patient and one patient with idiopathic T-lymphocytopenia. Lowering the TREC cut-off value or adjusting the screening algorithm would result in similar numbers of (non-)actionable secondary findings. Outcomes of combined second tier tests with adjustments in cut-off value or screening algorithms would be highly dependent on chosen cut-off values or normal ranges.

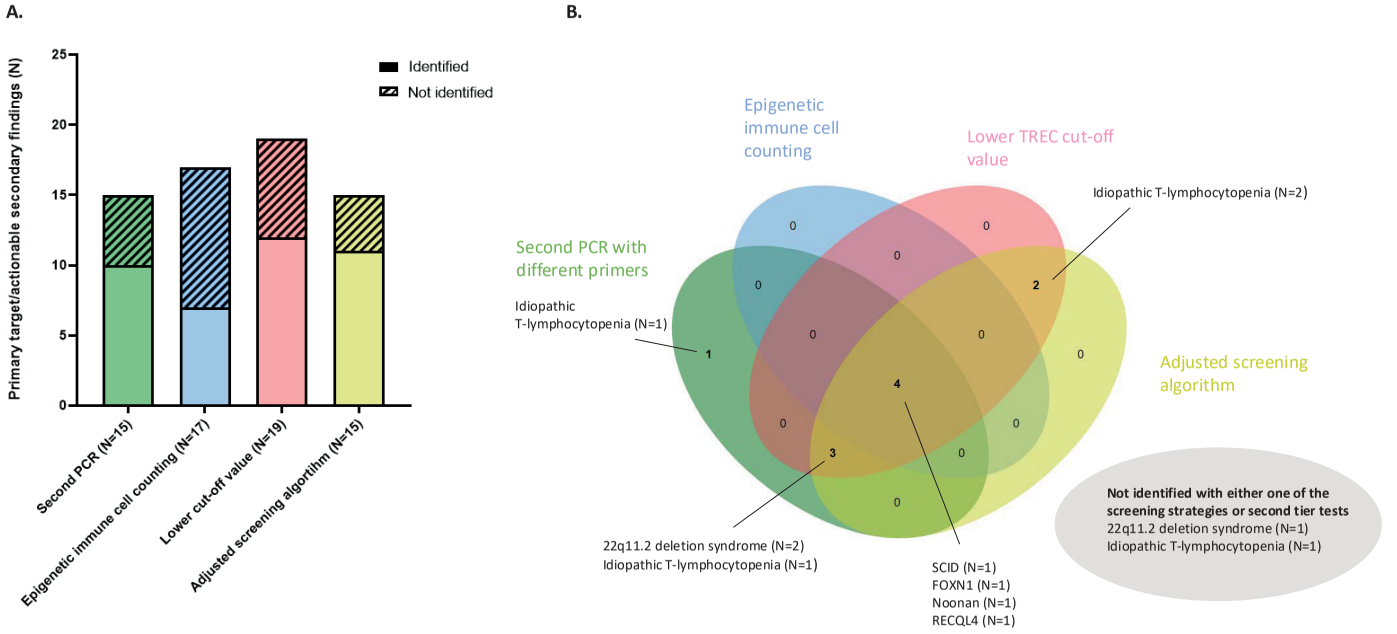

Figure 5. Effect of combined different screening strategies and second tier tests on number of referrals. **A**. Number of target disease (SCID) and actionable secondary findings identified/not identified per strategy. Not all referrals were analyzed with each screening strategy/second tier test. **B**. Overlap between the four different screening strategies in identifying/not identifying SCID and actionable secondary findings. Only actionable cases analyzed with all four screening strategies were included in the Venn diagram (N = 12). Two out of twelve referrals were not identified with any of the screening strategies

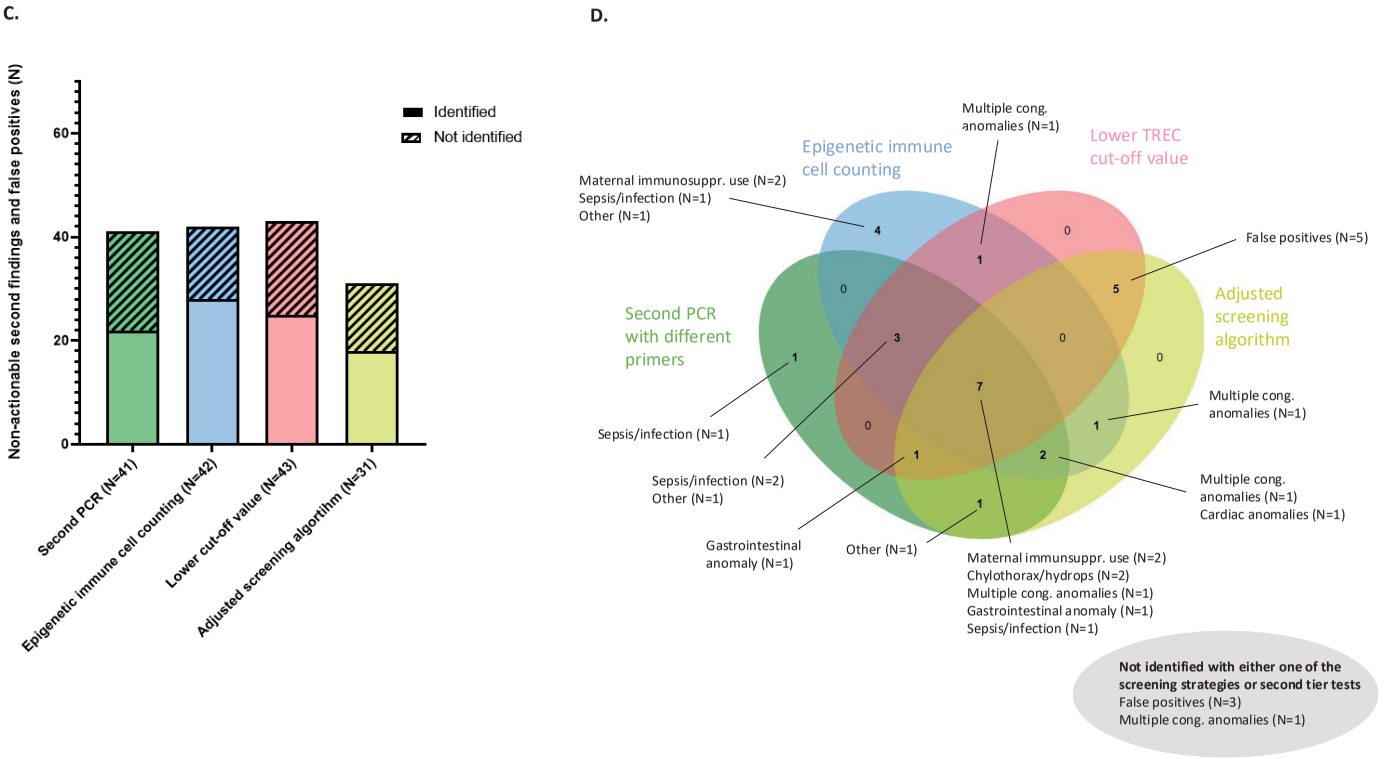

Figure 5. Continued. **C**. Number of non-actionable secondary findings and false positive cases identified/not identified per strategy. Not all referrals were analyzed with each screening strategy/second tier test. **D**. Overlap between the four different screening strategies in identifying and not identifying non-actionable secondary findings and false positive cases. Only non-actionable cases analyzed with all four screening strategies were included in the Venn diagram (N = 30). Four out of 30 referrals were not identified with any of the screening strategies [45].

DISCUSSION

Universal NBS for SCID was made possible by the development of a TREC assay utilizing DBS. TRECs are a highly sensitive biomarker for T-cell lymphopenia, but a non-specific marker for the primary target SCID, introducing the field of NBS to a palette of neonatal conditions and disorders associated with low T-cells around birth. NBS programs should make an effort to prevent non-actionable secondary findings and false-positive referrals that are associated with parental anxiety and costs for potentially unnecessary invasive tests. This study explored second tier test options after TREC analysis and adjustments in the TREC cut-off value and screening algorithm in order to reduce the number of referrals and emotional impact for parents and increase the positive predictive value for NBS for SCID.

Using a second PCR with different TREC primers has the potential to reduce the number of referrals, in particular the false-positive referrals with a SNP in the primer/probe region of the initial SPOT-it TREC assay. Not all laboratories will experience these primer/probe annealing problems due to SNPs, because that depends on the frequency of SNPs in the population and the chosen TREC assay or primer/probe combination. One has to keep in mind that each laboratory should determine their own cut-off values and that the referral rate will be highly depending on this chosen cut-off. This second tier test option is not limited to commercially available assays as screening laboratories can develop 'in house methods' with new primer sets. However, as some laboratories have strict criteria for accreditation and prefer using CE-IVD assays, the research-only NeoMDx assay is also available as a CE-IVD marked assay (EONIS SCID-SMA kit, PerkinElmer). Although PCR with different primers might not result in a much lower referral rate, this option does provide rapid availability of results with a feasible assay for any screening laboratory at relatively low costs.

Immunophenotyping and measuring absolute cell counts by flow cytometry is considered the golden standard in diagnostics for SCID. We showed that relative (epi) CD3+ T-cell counts measured with epigenetic qPCR had a much stronger correlation to absolute T-cell counts than TRECs (Pearson *r* correlation = 0.57 versus 0.86, $P < 0.001$), making it a more sensitive marker for T-cell lymphopenia. A potential improvement of the assay would be the identification of naïve T-cells. By including the measurement of naïve T-cells or recent thymic emigrants (RTEs) one could identify SCID cases with potentially maternal T-cell engraftment, or leaky SCID cases with oligoclonal T-cell expansion as seen in Omenn syndrome [2, 35]. A pitfall of measuring relative cell counts in contrast to absolute cell counts, is that proportional cell numbers within the corresponding reference range might not accurately reflect the clinically relevant

alterations in the patient. Patients could have very low numbers of total leukocytes with normal percentage of T-cells concealing a severe T-cell lymphopenia. Interestingly, epigenetic immune cell counting is not limited to measurement of CD3+ T-cells in DBS. In addition to SCID, there are many IEI that could benefit from early diagnosis and intervention if a suitable NBS test was available. With the Wilson and Jungner screening criteria in mind, some IEI might qualify for a disease that cause an important health problem and would benefit from early detection and treatment by preventing severe infections and auto-immunity [36]. With epigenetic immune cell counting, quantitative defects of other immune cell populations such as B-cells or neutrophils could offer early detection of X-linked agammaglobulinemia (XLA) and Severe Congenital Neutropenia (SCN) shortly after birth [34]. Automating the protocol would increase the throughput time for higher workloads, making epigenetic immune cell counting a valuable addition to future NBS for IEI.

Several countries have adjusted their cut-off value after pilot studies [11, 14, 24-26] or have implemented the request of a second NBS card for newborns with low TREC levels prior to referral into their screening algorithm [10, 28, 37]. Requesting a second NBS is based on the fact that TRECs can normalize in the first week(s) after birth in newborns with secondary T-cell impairment or in false-positive referrals. Lowering the cut-off value or requesting a second NBS card will not prevent the referral of false-positive cases with a SNP leading to amplification failure. In the Netherlands, requesting a second NBS card was introduced with national implementation of NBS for SCID on 1 January 2021. A distinction was made between 'TREC urgent positives' with absent/very low TREC levels and newborns with slightly higher, below cut-off TREC levels similar to the Polish/German trans-border cooperation for NBS for SCID [10]. The Israeli NBS program does not discriminate between TREC positive cases and requests a second NBS card for all newborns with TRECs below cut-off [37]. There is no international consensus on the time frame in which this repeated sampling should be performed, but a balance between time for TRECs to normalize and the risk of developing infections in newborns with severe T-cell problems should be pursued. In the Netherlands, a second confirmation NBS card is collected after seven days for newborns with TRECs between 2 - 10 copies/punch. In the coming years, more evidence on these screening algorithms, urgent positive TREC cut-off values and time frames for repeated sampling will be collected. Finally, we should acknowledge that repeated sampling is not without anxiety and emotional insecurity for parents and additional distress for the newborn. Implementing repeating sampling in any screening algorithm should be well-thought-out. Clear information provision for parents is of utmost importance in this process [38, 39].

It can be challenging to make clear statements about the palette of actionable and non-actionable secondary findings, as the exact case definition of actionable is not specified. One could argue that the term 'actionable' is mainly depending on the absolute T-cell numbers and the duration of the T-cell defect, but there is need for uniform case definitions across international NBS programs for SCID. Combining different screening strategies and second tier tests is not the preferred option for NBS programs. Multiple second tier tests would require more DBS material, which is not always available and might be required for other (more urgent) second tier NBS tests. In addition, a combination of screening strategies could introduce a significant delay in reporting the definitive screening results and referral of the newborn. The different screening strategies discussed in this paper show different numbers of identified and 'missed' actionable secondary findings (Figure 5, Table S1 and Table S2). From a clinical perspective, early diagnosis and management of actionable T-cell lymphopenia provide a clear and valuable health benefit for the child. However, NBS is aimed at detection of the primary target disease and from a public health perspective, programs aimed at neonatal screening should try to avoid secondary findings where possible. Opting for a test method with the lowest chance of secondary findings, regardless of their actionality, is the preferred option. Each public health program should take these different perspectives into account when the deciding on a balanced harm-benefit ratio for their NBS programs.

In addition to the options discussed in this study, other programs tried to reduce the number of secondary findings by implementing NGS as a second tier test [31, 32]. NGS with targeted gene panels on the initial NBS card will facilitate and accelerate final molecular diagnoses of affected newborns while providing useful information for management and follow-up. Targeted NGS has a rapid turn-around time and a higher TREC cut-off value in combination with NGS allows the detection of atypical and leaky SCID with clear HSCT indication [31]. However, it is important to note that if no pathogenic variants are identified with NGS, disease-causing variants could still be present in genes not included in the NGS gene panel. Moreover, structural and intronic variants can be missed with exon based targeted NGS. A 'safety net' including follow-up should be included for apparently healthy babies with low TRECs without pathogenic NGS findings. Genes included in the panels need to be constantly updated, a plan for managing 'variants of unknown significance' needs to be developed and functional validation assays are required to prove pathogenicity of novel variants [31]. NGS is associated with relatively high analyses and equipment costs and a cost-effectiveness analysis including efficiency gains and improved management could help NBS policy makers when discussing implementation of NGS [40]. The successful implementation of NGS in NBS as a second tier opens

discussion for expansion of NBS for immunodeficiencies by using NGS as a first tier [41, 42]. Sequencing without including any phenotypic markers as a first tier option remains challenging due to missing links between disease pathogenesis and gene expression and the inability to distinguish underlying pathogenic variants from the high number of genomic variations [5]. Overall, high throughput NGS analysis using targeted gene panels as a second tier after TREC analysis can reduce (false-positive) referrals while increasing the diagnostic precision and the specificity in NBS programs for SCID. Before implementation, any second tier test option should be evaluated in a broader perspective taking sensitivity, specificity, costs, feasibility for screening laboratories and throughput time into account.

In conclusion, second tier tests or adjustments in cut-off values and screening algorithms all have the potential to reduce the number of non-actionable secondary findings and false-positive referrals in NBS for SCID. A second PCR with different primers would prevent false-positive referrals caused by TREC amplification failure attributed to variations in the TREC primer/probe region. Epigenetic immune cell counting could also serve as a first tier in NBS for IEI if the protocol would be automated and throughput time increased. Rapid NGS seems to better fit the role of a second tier test, facilitating and accelerating molecular diagnoses of affected newborns. These findings will be of aid to any NBS program by attempting to prevent non-actionable secondary findings and false-positive referrals and increase the predictive value for NBS for SCID.

Acknowledgments

The authors would like to thank all those involved in the SONNET-study for their contribution and support. A special thanks to all members of the ANS-SCID committee for their suggestions and input during the study. The authors would like to express their gratitude to Mikael Hjort, Maija Siitonen and Henriikka Salomaki from PerkinElmer and to Epimune GmbH, in particular to Christoph Sachsenmaier for providing reagents free of charge and for the inspiring collaboration throughout the project.

DECLARATIONS

Funding
This study was funded by The Netherlands Organisation for Health Research and Development ZonMw (SONNET study, project 543002002).

Conflicts of interest/Competing interests
JS and JW are employees of the company Epimune GmbH. The other authors declare that they have no conflict of interest.

Availability of data and material
All data generated or analyzed during this study are included in this published article and its supplementary information.

Code availability
Not applicable

Authors' contributions
MB and MvdB designed the study; MB, IP, SI and LV performed analyses; RB did the clinical evaluations of the patients; MB, IP, SI, LV, JS and JW analyzed the data; MvdB coordinated the project; MB, RB and MvdB wrote the paper; all authors contributed to and approved the final version of the manuscript.

Ethics approval
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

Consent to participate
In order to participate in the SONNET-study, parents have to express verbal consent when the heel prick is performed (opt-out consent).

Consent for publication
The consent to participate included for publication of the data.

REFERENCES

1. Bousfiha, A., et al., Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. J Clin Immunol, 2020.
2. Fischer, A., et al., Severe combined immunodeficiencies and related disorders. Nat Rev Dis Primers, 2015. 1: p. 15061.
3. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
4. Pai, S.Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med, 2014. 371(5): p. 434-46.
5. Currier, R. and J.M. Puck, SCID newborn screening: What we've learned. Journal of Allergy and Clinical Immunology, 2021. 147(2): p. 417-426.
6. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol, 2005. 115(2): p. 391-8.
7. Kwan, A., et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama, 2014. 312(7): p. 729-38.
8. Chien, Y.-H., et al., Newborn Screening for Severe Combined Immunodeficiency in Taiwan. International Journal of Neonatal Screening, 2017. 3(3): p. 16.
9. van der Burg, M., et al., Universal Newborn Screening for Severe Combined Immunodeficiency (SCID). Front Pediatr, 2019. 7: p. 373.
10. Giżewska, M., et al., Newborn Screening for SCID and Other Severe Primary Immunodeficiency in the Polish-German Transborder Area: Experience From the First 14 Months of Collaboration. Front Immunol, 2020. 11: p. 1948.
11. Argudo-Ramírez, A., et al., First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain). Frontiers in immunology, 2019. 10: p. 2406-2406.
12. Hazenberg, M.D., et al., T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J Mol Med (Berl), 2001. 79(11): p. 631-40.
13. Buchbinder, D., et al., When Screening for Severe Combined Immunodeficiency (SCID) with T Cell Receptor Excision Circles Is Not SCID: a Case-Based Review. Journal of Clinical Immunology, 2021. 41(2): p. 294-302.
14. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2).
15. Mauracher, A.A., et al., Causes of low neonatal T-cell receptor excision circles: A systematic review. J Allergy Clin Immunol Pract, 2017. 5(5): p. 1457-1460.e22.
16. Thomas, C., et al., Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clin Immunol, 2019. 202: p. 33-39.
17. Blom, M., et al., Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J Clin Immunol, 2021. 41(1): p. 99-108.
18. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
19. Dorsey, M. and J. Puck, Newborn Screening for Severe Combined Immunodeficiency in the US: Current Status and Approach to Management. Int J Neonatal Screen, 2017. 3(2).

20. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. J Allergy Clin Immunol, 2017. 139(3): p. 733-742.
21. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunological reviews, 2019. 287(1): p. 241-252.
22. Tarini, B.A., The current revolution in newborn screening: new technology, old controversies. Arch Pediatr Adolesc Med, 2007. 161(8): p. 767-72.
23. Hewlett, J. and S.E. Waisbren, A review of the psychosocial effects of false-positive results on parents and current communication practices in newborn screening. J Inherit Metab Dis, 2006. 29(5): p. 677-82.
24. Audrain, M.A.P., et al., Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). J Clin Immunol, 2018. 38(7): p. 778-786.
25. Zetterström, R.H., et al., Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden. International Journal of Neonatal Screening, 2017. 3(2): p. 11.
26. Kanegae, M.P.P., et al., NEWBORN SCREENING FOR SEVERE COMBINED IMMUNODEFICIENCIES USING TRECS AND KRECS: SECOND PILOT STUDY IN BRAZIL. Rev Paul Pediatr, 2017. 35(1): p. 25-32.
27. Rechavi, E., et al., First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol, 2017. 8: p. 1448.
28. Trück, J., et al., Swiss newborn screening for severe T and B cell deficiency with a combined TREC/KREC assay - management recommendations. Swiss Med Wkly, 2020. 150: p. w20254.
29. Hale, J.E., et al., Ten Years of Newborn Screening for Severe Combined Immunodeficiency (SCID) in Massachusetts. J Allergy Clin Immunol Pract, 2021.
30. Routes, J. and J. Verbsky, Newborn Screening for Severe Combined Immunodeficiency. Current Allergy and Asthma Reports, 2018. 18(6): p. 34.
31. Strand, J., et al., Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol, 2020. 11: p. 1417.
32. Al-Mousa, H., et al., High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. Front Immunol, 2018. 9: p. 782.
33. Vogel, B.H., et al., Newborn screening for SCID in New York State: experience from the first two years. Journal of clinical immunology, 2014. 34(3): p. 289-303.
34. Baron, U., et al., Epigenetic immune cell counting in human blood samples for immunodiagnostics. Sci Transl Med, 2018. 10(452).
35. Kalina, T., et al., EuroFlow Standardized Approach to Diagnostic Immunopheneotyping of Severe PID in Newborns and Young Children. Front Immunol, 2020. 11: p. 371.
36. Wilson, J.M. and Y.G. Jungner. Principles and practice of screening for disease. World Health Organization 1968 Oct; 1968/10/01:[Available from: https://apps.who.int/iris/handle/10665/37650.
37. Rechavi, E., et al., Newborn Screening for Severe Combined Immunodeficiency in Israel. International Journal of Neonatal Screening, 2017. 3(2): p. 13.
38. Tu, W.-J., et al., Psychological effects of false-positive results in expanded newborn screening in China. PloS one, 2012. 7(4): p. e36235-e36235.

39. Gurian, E.A., et al., Expanded newborn screening for biochemical disorders: the effect of a false-positive result. Pediatrics, 2006. 117(6): p. 1915-21.
40. Berg, J.S., et al., Newborn Sequencing in Genomic Medicine and Public Health. Pediatrics, 2017. 139(2): p. e20162252.
41. King, J.R. and L. Hammarström, Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. Journal of clinical immunology, 2018. 38(1): p. 56-66.
42. Friedman, J.M., et al., Genomic newborn screening: public health policy considerations and recommendations. BMC Med Genomics, 2017. 10(1): p. 9.
43. Reference SNP (rs) Report rs377686467. 2020 April 21, 2020 [cited 2021 16 April]; Available from: https://www.ncbi.nlm.nih.gov/snp/rs377686467.
44. Reference SNP (rs) Report rs1466932014. 2020 April 21, 2020 [cited 2021 16 April]; Available from: https://www.ncbi.nlm.nih.gov/snp/rs1466932014.
45. Bardou, P., et al., jvenn: an interactive Venn diagram viewer. BMC Bioinformatics, 2014. 15(1): p. 293.

SUPPLEMENTARY MATERIAL

Table S1. Sub-diagnoses of (non-)actionable secondary findings that would be identified or not identified afterward second tier testing or screening algorithm adjustments.

	SONNET study	**PCR with different primers**		
	Identified *N*	Identified *N*	Not identified *N*	Not tested *N*
Target disease				
SCID (X-Linked)	1	1	0	0
Actionable secondary findings				
22q11.2 deletion syndrome	4	2	2	0
Trisomy 21	3	1	0	2
Noonan syndrome	1	1	0	0
Heterozygous FOXN1 variant	1	1	0	0
RECQL4 variant	1	1	0	0
RMRP variant	1	0	0	1
Idiopathic T-cell lymphocytopenia	7	3	3	1
Non-actionable secondary findings				
Multiple congenital anomalies [a]	6	2	4	0
Congenital diaphragmatic hernia	6	3	1	2
Cardiac anomalies	2	1	1	0
Gastrointestinal anomalies	2	2	0	0
Chylothorax and hydrops	3	3	0	0
Sepsis and severe infections	7	5	2	0
Maternal immunosuppr. use	4	2	2	0
Other neonatal conditions [b]	5	3	2	0
False-positives	8	0	8	0
Total (N)	**62**	**31**	**25**	**6**

a. Multiple congenital anomalies included newborns with nemaline rod myopathy (de novo variant ACTA1), holoprosencephaly/diaphragmatic hernia due to GLI1 variant, MADD deficiency and other defects.

b. Other neonatal conditions included severe asphyxia, dysmaturity, high doses of dexamethasone and start of chemotherapeutics prior to sample collection.

	Epigenetic immune cell counting			If a lower cut-off value was applied (TREC≤6 copies/punch)			If a new screening algorithm was applied [c]		
	Identified *N*	Not identified *N*	Not tested *N*	Identified *N*	Not identified *N*	Not included *N*	Identified *N*	Not identified *N*	Not included *N*
	1	0	0	1	0	0	1	0	0
	1	3	0	2	2	0	2	1	1
	2	0	1	1	2	0	1	0	2
	1	0	0	1	0	0	1	0	0
	0	1	0	1	0	0	1	0	0
	1	0	0	1	0	0	1	0	0
	1	0	0	1	0	0	1	0	0
	0	6	1	4	3	0	3	3	1
	5	1	0	2	4	0	3	2	1
	5	0	1	3	3	0	1	0	5
	2	0	0	0	2	0	1	0	1
	1	1	0	2	0	0	2	0	0
	3	0	0	3	0	0	2	0	1
	5	2	0	5	2	0	1	4	2
	4	0	0	2	2	0	2	2	0
	3	2	0	3	2	0	1	2	2
	0	8	0	5	3	0	5	3	0
	35	**24**	**3**	**37**	**25**	**0**	**29**	**17**	**16**

c. Number of (secondary) findings identified and not identified if a new screening algorithm was applied during the SONNET-study. Only newborns with TREC 0-2 (N = 14, SCID case included) and newborns with TREC measurements in peripheral blood (N = 32) were included.

Table S2. Referred newborns (N = 62) that are identified (+), not identified (-) or not tested (NT) after second tier testing or screening algorithm adjustments.

Patient number	Diagnosis	PCR with different primers	Epigenetic immune cell counting	If a lower cut-off value was applied (TREC≤ 6 copies/punch)	If a new screening algorithm was applied
1	SCID	+	+	+	+
2	22q11.2 deletion syndrome	+	-	+	+
3	22q11.2 deletion syndrome	+	-	+	+
4	22q11.2 deletion syndrome	-	-	-	-
5	22q11.2 deletion syndrome	-	+	-	NT
6	Trisomy 21	+	+	+	NT
7	Trisomy 21	NT	+	-	+
8	Trisomy 21	NT	-	-	NT
9	Noonan syndrome	+	+	+	+
10	Heterozygous FOXN1 variant	+	+	+	+
11	RECQL4 variant	+	+	+	+
12	RMRP variant	NT	+	+	+
13	Idiopathic T-cell lymphocytopenia	-	-	+	+
14	Idiopathic T-cell lymphocytopenia	-	-	+	+
15	Idiopathic T-cell lymphocytopenia	+	-	+	NT
16	Idiopathic T-cell lymphocytopenia	NT	NT	-	-
17	Idiopathic T-cell lymphocytopenia	+	-	-	-
18	Idiopathic T-cell lymphocytopenia	-	-	-	-
19	Idiopathic T-cell lymphocytopenia	+	-	+	+
20	Multiple congenital anomalies	+	+	+	+
21	Multiple congenital anomalies	+	+	-	+
22	Multiple congenital anomalies	-	+	-	+
23	Multiple congenital anomalies	-	-	-	-
24	Multiple congenital anomalies	-	+	+	-
25	Multiple congenital anomalies	-	+	-	NT
26	Congenital diaphragmatic hernia	NT	+	+	+
27	Congenital diaphragmatic hernia	+	+	+	NT

Table S2. Continued.

Patient number	Diagnosis	PCR with different primers	Epigenetic immune cell counting	If a lower cut-off value was applied (TRECs ≤ 6 copies/punch)	If a new screening algorithm was applied
28	Congenital diaphragmatic hernia	+	+	+	NT
29	Congenital diaphragmatic hernia	-	-	-	NT
30	Congenital diaphragmatic hernia	NT	+	-	NT
31	Congenital diaphragmatic hernia	+	+	-	NT
32	Cardiac anomalies	+	+	-	+
33	Cardiac anomalies	-	+	-	NT
34	Gastrointestinal anomalies	+	+	+	+
35	Gastrointestinal anomalies	+	-	+	+
36	Chylothorax and hydrops	+	+	+	+
37	Chylothorax and hydrops	+	+	+	+
38	Chylothorax and hydrops	+	+	+	NT
39	Sepsis and severe infections	+	+	+	+
40	Sepsis and severe infections	+	+	+	NT
41	Sepsis and severe infections	+	+	+	-
42	Sepsis and severe infections	+	+	+	-
43	Sepsis and severe infections	-	-	+	NT
44	Sepsis and severe infections	-	+	-	-
45	Sepsis and severe infections	+	-	-	-
46	Maternal immunosuppr. Use	+	+	+	+
47	Maternal immunosuppr. Use	+	+	+	+
48	Maternal immunosuppr. use	-	+	-	-
49	Maternal immunosuppr. use	-	+	-	-
50	Other neonatal condition	+	-	-	+
51	Other neonatal condition	+	+	+	-
52	Other neonatal condition	+	-	+	NT
53	Other neonatal condition	-	+	+	NT
54	Other neonatal condition	-	+	-	-
55	False-positive	-	-	+	+

Table S2. Continued.

Patient number	Diagnosis	PCR with different primers	Epigenetic immune cell counting	If a lower cut-off value was applied (TREC≤ 6 copies/punch)	If a new screening algorithm was applied
56	False-positive	-	-	+	+
57	False-positive	-	-	+	+
58	False-positive	-	-	+	+
59	False-positive	-	-	+	+
60	False-positive	-	-	-	-
61	False-positive	-	-	-	-
62	False-positive	-	-	-	-

CHAPTER 6

Abnormal results of newborn screening for SCID after azathioprine exposure *in utero*: benefit of *TPMT* genotyping in both mother and child

Maartje Blom, Ingrid-Pico Knijnenburg, Joris van Montfrans, Robbert Bredius, Mirjam van der Burg, Jesse Swen and Dagmar Berghuis

Journal of Clinical Immunology, 2021; Online ahead of print

TO THE EDITOR

INTRODUCTION

Countries all over the world are progressively implementing newborn screening (NBS) for severe combined immunodeficiency (SCID). NBS for SCID is based on the detection of T-cell receptor excision circles (TRECs), a marker for thymic production of naïve T-cells. However, newborns with a range of conditions associated with T-cell lymphopenia (including other conditions than SCID) are being identified shortly after birth. Due to the global rollout of NBS for SCID, pediatric immunologists are confronted with numerous and various (neonatal) cases of impaired T-cell development. One of the causes of profound T-cell lymphopenia encountered in NBS for SCID is the maternal use of immunosuppressive drugs such as azathioprine during pregnancy [1-6].

Azathioprine is an immunosuppressive cytotoxic drug used for treatment of several autoimmune disorders, including inflammatory bowel disease (IBD). Azathioprine is a prodrug, rapidly metabolized to active 6-thiogunine nucleotides [6-TGN], that are incorporated in the DNA inhibiting purine synthesis and thus cause cell cytotoxicity [7]. Azathioprine is often prescribed during pregnancy to women with IBD to avoid flares and relapse of the disease. Exacerbations of disease activity are associated with an increased risk of pre- and/or dysmaturity. The use of azathioprine during pregnancy is considered relatively safe, however, cases of hematological toxicity and neonatal immunodeficiency have been reported [8].

Azathioprine toxicity (including severe lymphopenia) has been attributed to genetic polymorphisms in the *TPMT* gene, which is responsible for enzymatic catalyzation of azathioprine to the inactive metabolite 6-MMP. Presence of non-functional *TPMT* alleles results in reduced TPMT activity and, thereby, accumulation of active 6-TGN and increased toxicity. TPMT phenotyping and/or genotyping allows individualized azathioprine dosing in patients with either one non-functional *TPMT* allele ('intermediate metabolizers'; prevalence 6 - 11% in Caucasian populations) or two non-functional TPMT alleles ('poor metabolizers'; prevalence 0.3% in Caucasian populations). Guidelines for *TPMT*-informed dosing of azathioprine are available and could prevent toxicity in the newborn [9,10].

Here, we describe four cases of newborns with significant combined T- and B-cell lymphopenia, identified via NBS for SCID, born to mothers using azathioprine. We highlight the case of a girl who was referred to a pediatrician-immunologist with severe T-cell lymphopenia, requiring infection prophylaxis. The T-cell lymphopenia

was caused by *in utero* exposure to high levels of azathioprine/6-TGN due to strongly reduced TPMT enzyme activity. In addition, we report three other cases with significant T-cell lymphopenia after maternal azathioprine use identified by NBS for SCID. All parents provided consent for participation in NBS for SCID and consent for publication.

CASE DESCRIPTION

Maternal history

A 37 year old patient (G2P2) with IBD, was treated with azathioprine 100mg per day throughout pregnancy. No recent 6-TGN levels were known and no *TPMT* genotyping was performed. During pregnancy, a complete blood count (without differential count) showed no anemia, thrombocytopenia or leukopenia. She gave birth to a daughter after an uncomplicated pregnancy (*case A*, see below).

Four weeks after delivery, a differential blood count, 6-TGN/6-MMP levels and TPMT genotyping were performed because of lymphopenia in her newborn daughter. Differential showed lymphopenia (lymphocytes 0.5 x 109/L), high 6-TGN levels (647 pmol/8x108 RBC) with undetectable 6-MMP levels and a heterozygous *3C *TPMT* genotype (intermediate metabolizer).

Patient history (*case A*)

A newborn girl, second child of non-consanguineous parents, was born at 39 4/7 weeks with normal birth weight (3245 grams). She was not breastfed. NBS for SCID was abnormal at day 4 (TRECs 0 copies/punch) and she was referred to the pediatrician-immunologist for further analysis. There were no clinical abnormalities. A differential blood count at day 7 showed no anemia nor neutropenia, but low thrombocytes (52 x 109/L) and severe lymphopenia (0.26 x 109/L). Lymphocyte subset analysis revealed a SCID-like phenotype with (near-)absent T- and B-cells (Figure 1A). Epigenetic immune cell counting (technique for relative leukocyte quantification in dried blood spots [11]) confirmed low relative T- and B-cell counts in the original NBS card. 6-TGN levels were high (618 pmol/8x108 RBC), 6-MMP levels were undetectable. Because of the severe T-lymphopenia, Pneumocystis prophylaxis (trimethoprim-sulphamethoxazole) was started at day 7 and home isolation was advised. Genetic analysis revealed a homozygous *3C *TPMT* genotype (poor metabolizer). The clinical condition of the patient, lymphocyte counts (including TREC analysis) and 6-TGN/6-MMP levels were regularly assessed and normalized during follow-up (Figure 1A-C). Pneumocystis prophylaxis and home isolation were discontinued at week eight (based on CD3+/CD4+ T-cell counts) and the patient was discharged from further follow-up at 18 weeks.

Table 1. Diagnostic results of case A; Girl, 2nd child of non-consanguineous parents, gestational age 39 4/7 weeks, birth weight 3245 grams. Maternal azathioprine use (1d 100 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 5	Week 8	Week 18	Normal range
Blood cell counts						
Hemoglobin (Hb) (mmol/L)	-	9.1	7.1	6.3	6.9	6.5 - 8.4
Thrombocytes (x10^9/L)	-	52	337	443	426	150 – 450
Leukocytes (x10^9/L)	-	7.30	3.20	5.10	6.80	6.0 - 17.5
Neutrophils (x10^9/L)	-	5.30	1.50	1.70	2.00	1.5 - 8.5
Flow cytometry						
Lymphocytes (x10^9/L)	-	0.26	0.64	2.31	3.7	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	37	73	866	2575	2300 -7000
CD4+ T-cells (x 10^6/L)	-	31	60	735	2000	1700 -5300
CD4+ naïve T-cells (x 10^6/L)		13	37	606	1800	
CD8+ T-cells (x 10^6/L)	-	4	0	69	434	394 – 1865
CD8+ naïve T-cells (x 10^6/L)		2	0	53	361	
CD19+ B cells (x 10^6/L)	-	0	464	1133	913	600 – 1900
CD56+ NK cells (x 10^6/L)	-	193	52	249	178	200 – 1400
Ig analysis						
IgG (g/L)	-	9.1	6.1	4.1	2.2	2.20 - 11.3
IgA (g/L)	-	-	0.00	0.00	0.08	0.080 - 0.90
IgM (g/L)	-	-	0.02	0.13	0.28	0.070 - 0.65
Epigenetic immune cell counting*						
Relative CD3+ T-cell counts (%)	0.90	1.59	-	15.15	36.45	11.32 - 34.95
Relative B-cell counts (%)	0.05	0.36	-	21.41	18.28	2.28 - 9.36
Relative NK-cell counts (%)	2.75	4.21	-	7.61	3.32	4.18 - 12.21
Screening results and 6-TGN/6-MMP levels						
TREC copies/punch	0 - 0 - 0	0 - 1 - 1	-	10-18-15	139-147-151	>10
B-actin copies/punch (average)	4369	5067	-	3348	3410	>1000
6-TGN pmol/8.10^8 RBC	-	618	126	0	-	Toxic range >450
6-MMP levels pmol/8.10^8 RBC	-	0	0	0	-	
Genetic analysis	*TPMT* genotype: homozygous *3C/*3C. Poor metabolizer.					

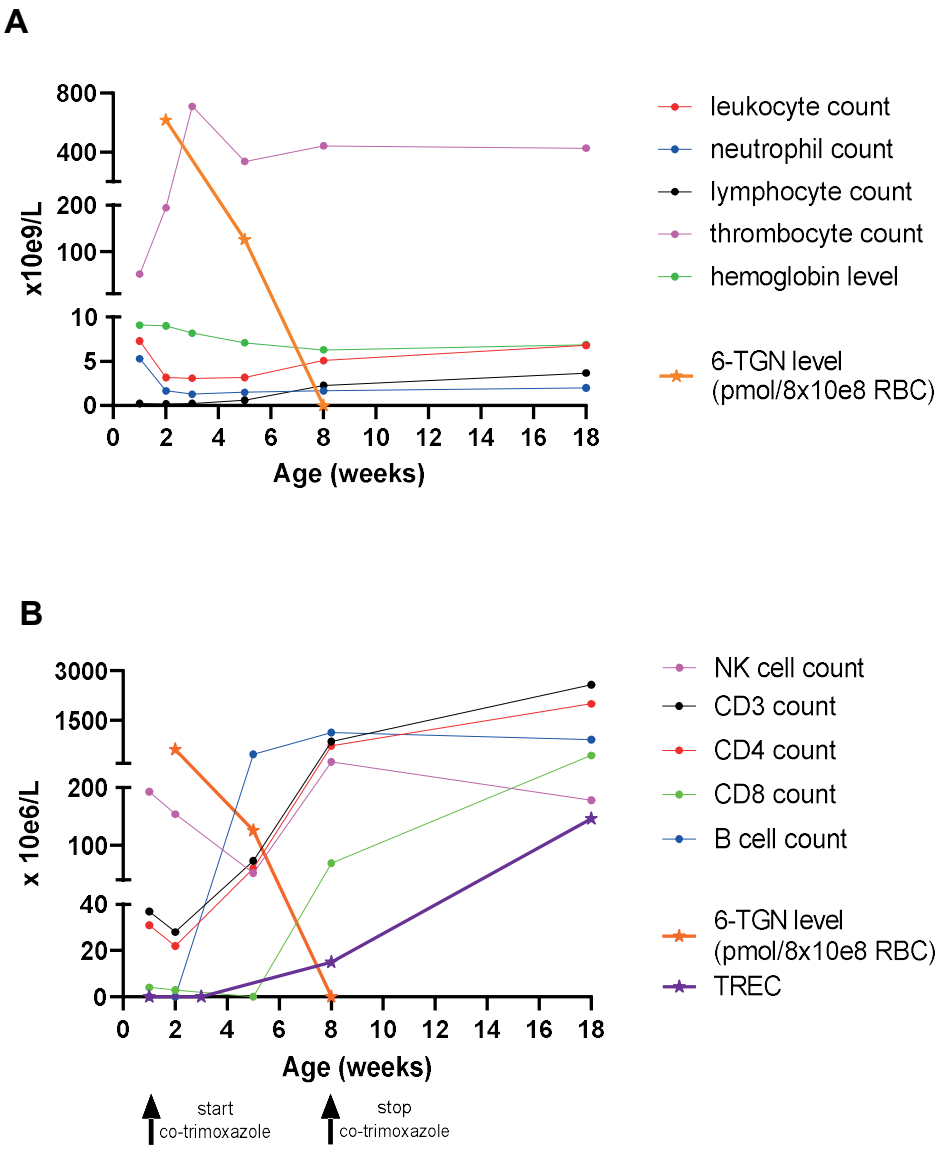

Figure 1. Diagnostic and screening results of case A. **A.** Absolute cell counts, hemoglobin level and 6-TGN levels over time. **B.** Absolute lymphocyte subset cell counts, TRECs and 6-TGN levels over time.

Sister of case A

Because of lymphopenia in *case A*, a retrospective analysis was performed on the original NBS card of her older sister (two years of age). During this pregnancy, mother used azathioprine 50 mg once daily. This healthy sister appeared to have normal TREC levels (26 copies/punch) and normal relative epigenetic CD3+ T-cell counts (18.0%, normal range 11.3 - 35.0%) in her NBS card. Relative epigenetic B-cell counts were very low 0.16% (normal range 2.28 - 9.36). *TPMT* genotyping showed this sister to be an intermediate metabolizer (heterozygous *TPMT* *3C allele).

Additional cases

Three other cases (*cases B-D*) with maternal azathioprine use were identified with NBS for SCID and referred to a pediatrician-immunologist in the same period. TREC counts in these patients varied between 1 and 16 copies/punch. All cases had profound combined T- and B-cell lymphopenia at time of referral and were monitored for up to 18 weeks, until immunological recovery (Figure S1, S2 and S3). Additional *TPMT* genotyping revealed that *case B* was a normal metabolizer (*TPMT* wild type genotype) but exposed to a relatively high dose of azathioprine (200 mg/day). *Case C* was a poor metabolizer (homozygous of *TPMT* *3A allele), exposed to a relatively low dose of azathioprine (75 mg/day). *Case D* was an intermediate metabolizer (heterogeneous for at least one *TPMT* *3 allele) exposed to 100 mg azathioprine/day. Because of low CD3+/CD4+ T-cell counts, *case D* received Pneumocystis prophylaxis until immunological recovery.

DISCUSSION

We describe four cases of newborns with profound combined T- and B-cell lymphopenia, identified via NBS for SCID, born to mothers using azathioprine. *TPMT* genotyping provided valuable additional information during the diagnostic process of these infants with severe lymphopenia, as azathioprine is frequently prescribed during pregnancy and not all newborns from mothers using this drug are affected. Both mothers and newborns with reduced TPMT enzyme activity caused by polymorphisms in the *TPMT* gene had less efficient catalyzation of azathioprine leading to a higher risk of hematopoietic toxicity, including profound lymphopenia (mimicking SCID) in the newborn. Two children with reduced TPMT enzyme activity and severe T-cell lymphopenia even required home isolation and initiation of Pneumocystis prophylaxis. All cases demonstrated complete immunological recovery at 10-18 weeks after birth.

Based on current international guidelines, health care providers prescribing azathioprine to pregnant women usually perform a total leukocyte count without differential, which may leave maternal lymphopenia due to reduced TPMT activity with toxic 6-TGN levels unnoticed [12]. This may result in provision of suboptimal information to (future) parents about the possibility of immunodeficiency and an abnormal result of NBS for SCID in the newborn. Indeed, not all families of our cases were aware of the risk associated with azathioprine use during pregnancy, including the possibility of abnormal NBS results. Parents were given conflicting information during the referral procedure after the abnormal SCID screening result. We earlier reported that referrals in NBS for SCID caused considerable anxiety in parents [13]. In addition, health care providers prescribing azathioprine to the mothers questioned the association between the abnormal NBS result and maternal azathioprine treatment.

With the global rollout of NBS for SCID, there is a strong need to raise awareness on a multidisciplinary scale about maternal azathioprine use and the risk of severe neonatal T-cell lymphopenia with abnormal SCID screening results. More explicit monitoring of maternal lymphocyte counts, 6-TGN/6-MMP levels and *TPMT* genotyping at the start of pregnancy, with adjustment of azathioprine dose without reducing therapeutic efficiency in mothers, may prevent fetal exposure to azathioprine toxicity *in utero*. Moreover, differential blood count analysis in (at-risk) newborns directly after birth may identify these cases prior to NBS for SCID. Maternal and patient history plus laboratory results of both mother and child, will additionally help will help pediatrician-immunologists in the evaluation of these newborns with abnormal SCID screening results. The provision of clear information by the health care providers involved, both during the pregnancy as well as during the referral procedure, is of utmost importance and can severely reduce anxiety for parents [13].

Sharing experiences of cases with profound lymphopenia, identified via NBS, with obstetricians, gastroenterologists, pediatricians and primary health care providers, and a close partnership between physicians on an international level, will help to promote standardization of care for fertile/pregnant women on immunosuppressant medication including azathioprine.

Acknowledgments
The authors would like to thank all those involved in the SONNET-study for their contribution to the study. A special thanks to all parents of the cases for their participation and support.

DECLARATIONS

Funding
The Netherlands Organisation for Health Research and Development ZonMw financed the SONNET-study (project 543002002).

Conflicts of interest/Competing interests
The authors declare that they have no conflict of interest.

Availability of data and material
All data generated or analyzed during this study are included in this published article and its supplementary information.

Code availability
Not applicable

Authors' contributions
MB, MvdB and DB designed the study; IP and JS performed flow cytometric and genetic analyses; DB, RB and JvM did the clinical evaluations of the patients; MB, IP, JS, DB analyzed the data; DB coordinated the project; MB and DB wrote the paper; all authors contributed to and approved the final version of the manuscript.

Ethics approval
This study was performed in line with the principles of the Declaration of Helsinki. Approval for the SONNET-study was granted by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

Consent to participate
In order to participate in newborn screening for SCID (SONNET-study), parents have to express verbal consent when the heel prick is performed (opt-out consent).

Consent for publication
Parents of cases have provided consent for the publication of the data in this case report.

REFERENCES

1. Thomas, C., et al., A Severe Neonatal Lymphopenia Associated With Administration of Azathioprine to the Mother in a Context of Crohn's Disease. Journal of Crohn's and Colitis, 2018. 12(2): p. 258-261.
2. Kuo, C.Y., et al., Profound T-cell lymphopenia associated with prenatal exposure to purine antagonists detected by TREC newborn screening. The Journal of Allergy and Clinical Immunology: In Practice, 2017. 5(1): p. 198-200.
3. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
4. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2): p. e20182300.
5. Thomas, C., et al., Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clinical Immunology, 2019. 202: p. 33-39.
6. Giżewska, M., et al., Newborn Screening for SCID and Other Severe Primary Immunodeficiency in the Polish-German Transborder Area: Experience From the First 14 Months of Collaboration. Frontiers in immunology, 2020. 11: p. 1948-1948.
7. Gearry, R.B. and M.L. Barclay, Azathioprine and 6-mercaptopurine pharmacogenetics and metabolite monitoring in inflammatory bowel disease. J Gastroenterol Hepatol, 2005. 20(8): p. 1149-57.
8. Akbari, M., et al., Systematic Review and Meta-analysis on the Effects of Thiopurines on Birth Outcomes from Female and Male Patients with Inflammatory Bowel Disease. Inflammatory Bowel Diseases, 2012. 19(1): p. 15-22.
9. Colombel, J.f., et al., Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. Gastroenterology, 2000. 118(6): p. 1025-1030.
10. Relling, M.V., et al., Clinical Pharmacogenetics Implementation Consortium Guideline for Thiopurine Dosing Based on TPMT and NUDT15 Genotypes: 2018 Update. Clin Pharmacol Ther, 2019. 105(5): p. 1095-1105.
11. Baron, U., et al., Epigenetic immune cell counting in human blood samples for immunodiagnostics. Sci Transl Med, 2018. 10(452).
12. van der Woude, C.J., et al., The Second European Evidenced-Based Consensus on Reproduction and Pregnancy in Inflammatory Bowel Disease. Journal of Crohn's and Colitis, 2015. 9(2): p. 107-124.
13. Blom, M., et al., Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J Clin Immunol, 2021. 41(1): p. 99-108.

SUPPLEMENTARY MATERIAL

Table S1. Diagnostic results of Case B; Boy, 1st child of non-consanguineous parents, gestational age 37.2 weeks, birthweight 3170 grams. Maternal azathioprine use (1d 200 mg) for inflammatory bowel disease. 6-TGN/6-MMP-levels in mother were 526 and 814 pmol/8.10^8 RBC respectively at three months of pregnancy.

	Day 4	Week 1	Week 10	Normal range
Blood cell counts				
Hemoglobin (Hb) (mmol/L)	-	9.2	6.0	6.5 - 8.4
Thrombocytes (x10^9/L)	-	354	536	150 - 450
Leukocytes (x10^9/L)	-	4.30	4.51	6.0 - 17.5
Neutrophils (x10^9/L)	-	2.38	1.15	1.5 - 8.5
Flow cytometry				
Lymphocytes (x10^9/L)	-	0.76	2.25	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	660	1314	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	404	990	1700 - 5300
CD4+ naïve T-cells (x 10^6/L)	-	411	883	
CD8+ T-cells (x 10^6/L)	-	106	272	394 - 1865
CD8+ naïve T-cells (x 10^6/L)	-	91	255	
CD19+ B cells (x 10^6/L)	-	0	718	600 - 1900
CD56+ NK cells (x 10^6/L)	-	68	126	200 - 1400
Immunoglobulin (Ig) analysis				
IgG (g/L)	-	5.8**	2.8	2.20 - 11.3
IgA (g/L)	-	0	0.06	0.080 - 0.90
IgM (g/L)	-	0.01	0.2	0.070 - 0.65
Epigenetic immune cell counting*				
Relative CD3+ T-cell counts (%)	7.05	14.40	-	11.32 - 34.95
Relative B-cell counts (%)	0.15	0.39	-	2.28 - 9.36
Relative NK-cell counts (%)	1.47	2.25	-	4.18 - 12.21
Screening results and 6-TGN levels				
TREC copies/punch	4 - 10 - 16	23 - 18 - 15	-	>10
B-actin copies/punch (average)	3589	1078	-	>1000
6-TGN pmol/8.10^8 RBC	-	< 50	-	Toxic range >450
Genetic analysis	TPMT genotype: 'wild type', normal metabolizer (no variants in the TPMT*2, *3A, *3B or *3C allele).			

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. *Relative cell counts in percentages (%) of total leukocytes. ** Week 3 measurements.

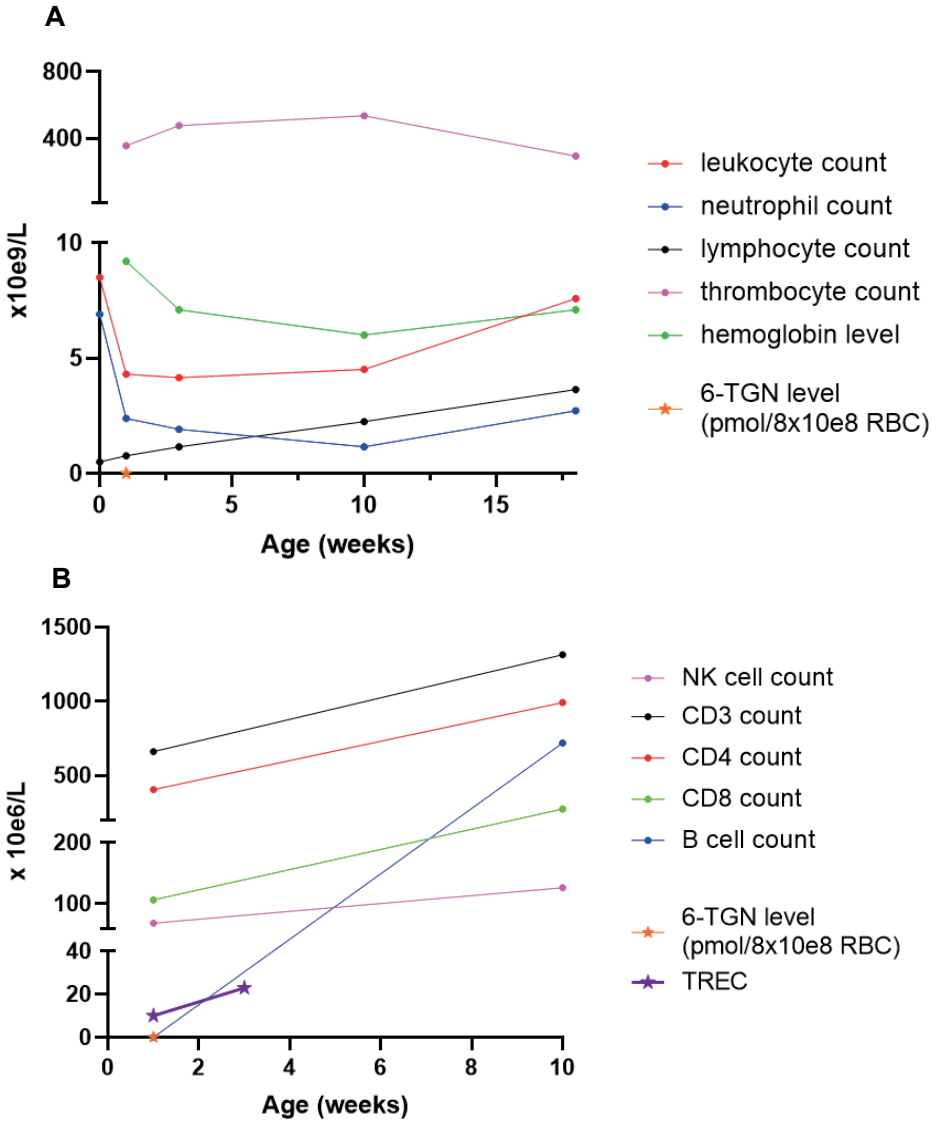

Figure S1. Diagnostic and screening results of case B. **A.** Absolute cell counts, hemoglobin level and 6-TGN levels over time. **B.** Absolute lymphocyte subset cell counts, TRECs and 6-TGN levels over time.

Table S2. Diagnostic results of Case C; Boy, 4th child of non-consanguineous parents, gestational age 40.1 weeks, birthweight 3032 grams. Maternal azathioprine use (1d 75 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 10	Normal range
Blood cell counts				
Hemoglobin (Hb) (mmol/L)	-	10.2	6.2	6.5 - 8.4
Thrombocytes (x10^9/L)	-	258	402	150 - 450
Leukocytes (x10^9/L)	-	4.30	5.80	6.0 - 17.5
Neutrophils (x10^9/L)	-	1.86	1.49	1.5 - 8.5
Flow cytometry				
Lymphocytes (x10^9/L)	-	0.60	3.43	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	720	1406	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	580	1101	1700 - 5300
CD4+ naïve T-cells (x 10^6/L)	-	-	909	
CD8+ T-cells (x 10^6/L)	-	80	216	394 - 1865
CD8+ naïve T-cells (x 10^6/L)	-	-	188	
CD19+ B cells (x 10^6/L)	-	0	1845	600 - 1900
CD56+ NK cells (x 10^6/L)	-	10	96	200 - 1400
Immunoglobulin (Ig) analysis				
IgG (g/L)	-	10.9	-	2.20 - 11.3
IgA (g/L)	-	0	-	0.080 - 0.90
IgM (g/L)	-	0.01	-	0.070 - 0.65
Epigenetic immune cell counting*				
Relative CD3+ T-cell counts (%)	4.61	13.18	-	11.32 - 34.95
Relative B-cell counts (%)	0.24	0.56	-	2.28 - 9.36
Relative NK-cell counts (%)	1.56	3.30	-	4.18 - 12.21
Screening results				
TREC copies/punch	7 - 3 - 6	11 - 18 - 16	-	>10
B-actin copies/punch (average)	4137	7240	-	>1000
Genetic analysis	TPMT genotype: homozygous *3A/*3A. Poor metabolizer.			

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. Relative cell counts in percentages (%) of total leukocytes.

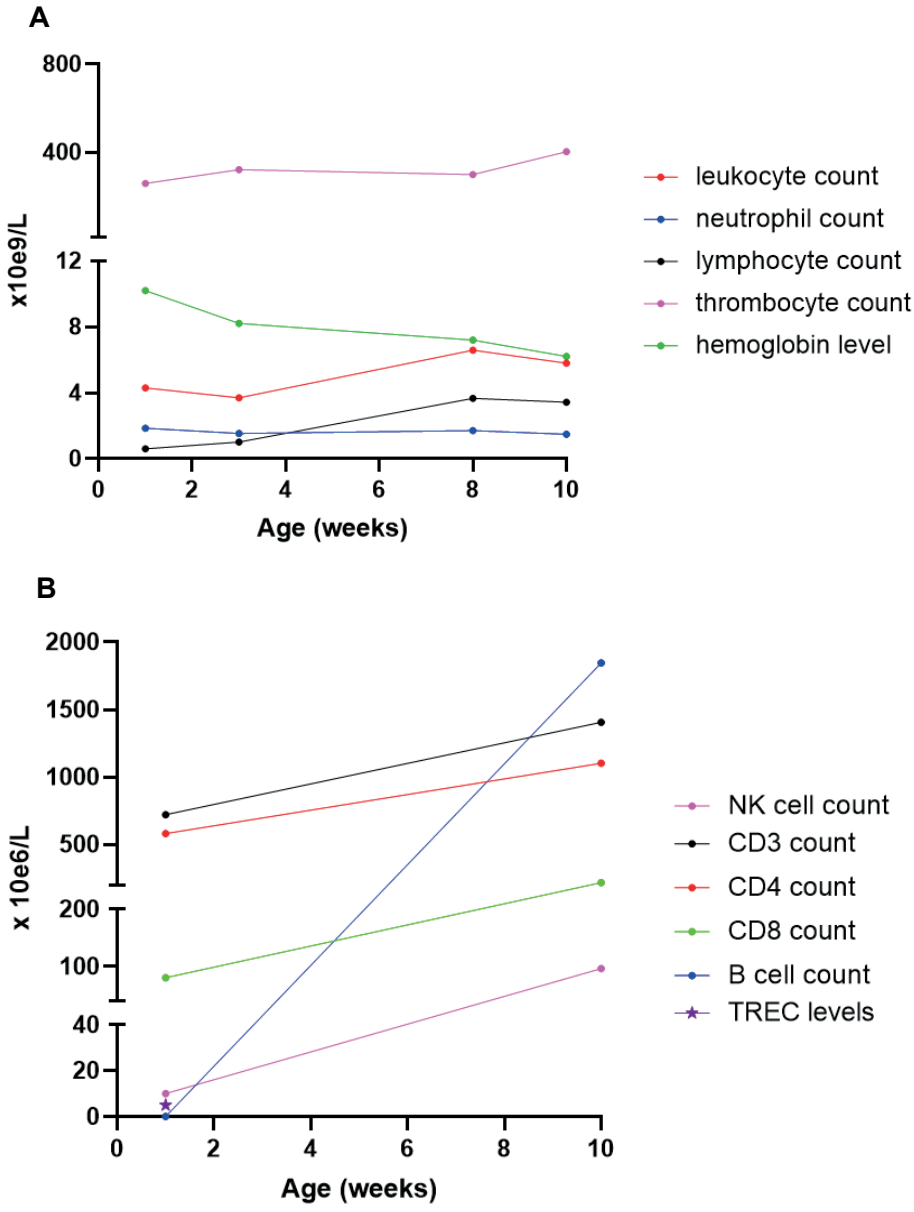

Figure S2. Diagnostic and screening results of case C. **A.** Absolute cell counts and hemoglobin level over time. **B.** Absolute lymphocyte subset cell counts and TREC levels over time.

Table S3. Diagnostic results of Case D; Boy, 1st child of non-consanguineous parents, gestational age 40.4 weeks, birthweight 3840 grams. Maternal azathioprine use (1d 100 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 6	Week 12	Week 26	Normal range
Blood cell counts						
Hemoglobin (Hb) (mmol/L)	-	10.6	7.3	6.4	7.0	6.5 - 8.4
Thrombocytes (x10^9/L)	-	301	549	378	570	150 - 450
Leukocytes (x10^9/L)	-	7.0	5.1	6.6	11.9	6.0 - 17.5
Neutrophils (x10^9/L)	-	4.07	1.88	2.49	2.74	1.5 - 8.5
Flow cytometry						
Lymphocytes (x10^9/L)	-	0.70	1.98	3.15	8.33	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	383	872	1961	4491	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	209	667	1538	3262	1700 - 5300
CD4+ naïve T-cells	-	133	489	1307	2734	
CD8+ T-cells (x 10^6/L)	-	101	170	310	1165	394 - 1865
CD8+ naive T-cells	-	88	120	217	860	
CD19+ B cells (x 10^6/L)	-	1	625	965	2146	600 - 1900
CD56+ NK cells (x 10^6/L)	-	68	392	134	370	200 - 1400
Immunoglobulin (Ig) analysis						
IgG (g/L)	-	7.3	-	2.37	3.58	2.20 - 11.3
IgA (g/L)	-	<0.04	-	0.05	0.21	0.080 - 0.90
IgM (g/L)	-	<0.04	-	1.1	0.50	0.070 - 0.65
Epigenetic immune cell counting*						
Relative CD3+ T-cell counts (%)	8.11	12.64	16.24	-	28.99	11.32 - 34.95
Relative B-cell counts (%)	0.11	0.09	12.9	-	18.0	2.28 - 9.36
Relative NK-cell counts (%)	1.30	3.47	4.72	-	2.96	4.18 - 12.21
Screening results						
TREC copies/punch	1 - 3 - 5	6 - 4 - 9	14-16-23	-	66	>10
B-actin copies/punch (average)	1882	2886	1648	-	1376	>1000
Genetic analysis	TPMT genotype: heterogenous for at least one *3 allele. Based on allele frequency most probable *1/*3A genotype. Intermediate metabolizer.					

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. Relative cell counts in percentages (%) of total leukocytes.

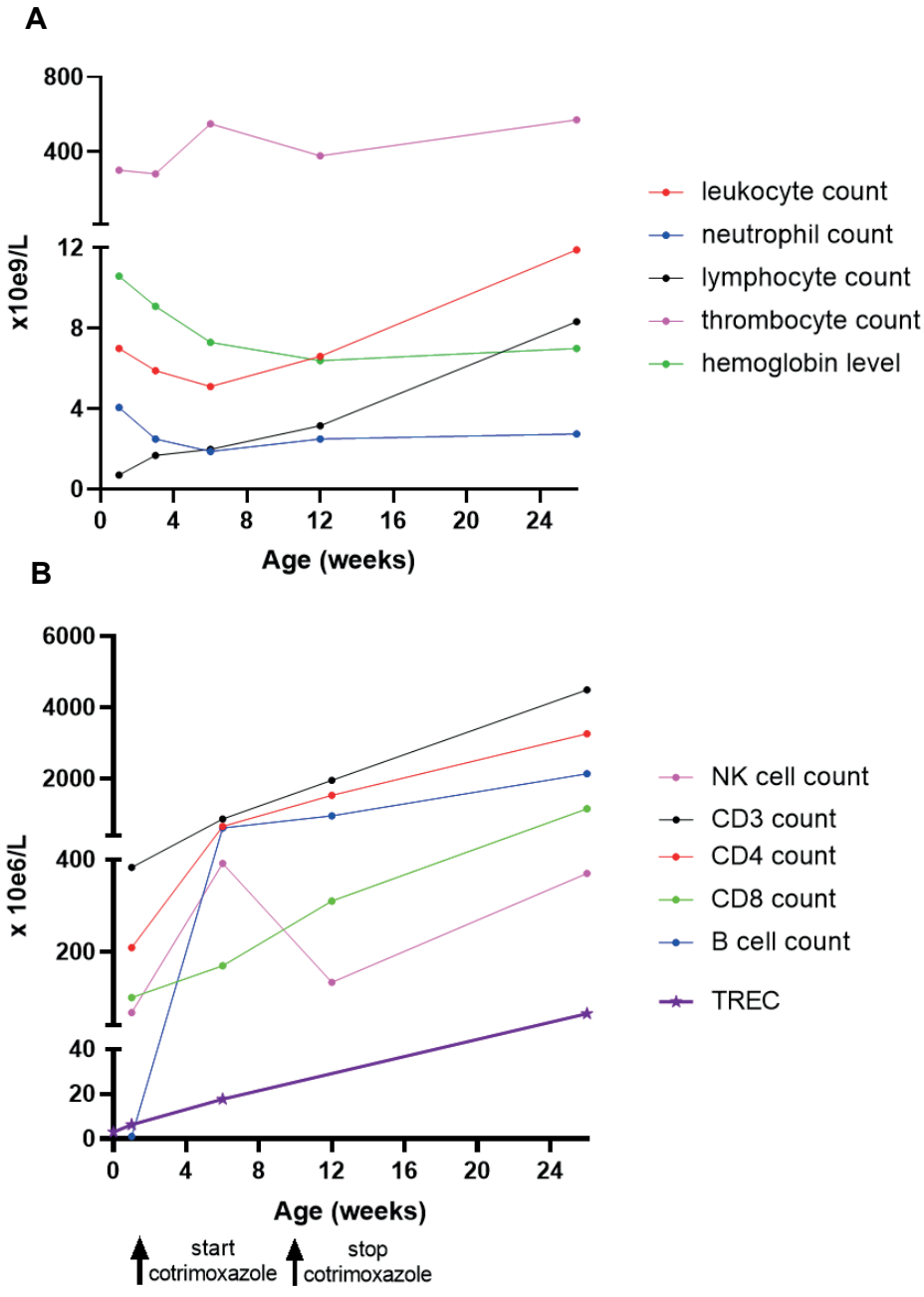

Figure S3. Diagnostic and screening results of case D. **A.** Absolute cell counts and hemoglobin level over time. **B.** Absolute lymphocyte subset cell counts and TREC levels over time.

CHAPTER 7

Early diagnosis of ataxia telangiectasia in the neonatal phase: a parents' perspective

Michiel H. D. Schoenaker, Maartje Blom, Martine C. de Vries, Corry M. R. Weemaes, Mirjam van der Burg and Michèl. A. A. P. Willemsen

European Journal of Pediatrics. 2020; 179(2): 251-256

ABSTRACT

Ataxia telangiectasia (A-T) is a severe neurodegenerative disorder with variable immunodeficiency. Together with the Dutch A-T community, we investigated the opinion of A-T parents on an early A-T diagnosis in the asymptomatic phase of the disease. During an annual national meeting for A-T patients and families, the topic of an early A-T diagnosis was discussed in relation to the recent introduction of neonatal screening for severe combined immunodeficiency (SCID) in the Netherlands. Based on the discussion, individual arguments were identified and processed into a questionnaire, which was sent out to 64 A-T parents (32 families). Arguments included were insecurity to diagnosis, possible medical advantages, appropriate genetic counseling and family planning, loss of "golden" year(s), and early cancer screening for parents. The response rate was 55% (N = 35 parents). Twenty-six (74%) parents felt that the advantages of an early diagnosis outweighed the disadvantages, five parents thought that the disadvantages would outweigh the advantages (14%), and four parents did not indicate a preference.

Conclusion

The majority of parents of a child with A-T would have preferred an early diagnosis during the asymptomatic phase of the disease, because the uncertainty during the diagnostic process had had a major impact on their lives. In addition, the knowledge of being carriers of an *ATM* gene mutation influenced decisions about family planning. Parents who opposed against an early diagnosis emphasized the joy of having a seemingly healthy child until diagnosis.

INTRODUCTION

Ataxia Telangiectasia (A-T) is a devastating, autosomal recessively inherited disease with a huge impact on quality of life of patients and their parents. A-T is a DNA repair disorder, caused by mutations in the *Ataxia Telangiectasia Mutate*d (*ATM*) gene, leading to neurodegeneration with progressive ataxia, telangiectasia's, predisposition to malignancies, sensitivity to radiation and immunodeficiency [1]. Patients with classic A-T have no symptoms in the first year of life; progressive symptoms, however, start shortly thereafter. An early diagnosis helps to start up A-T specialized care regarding the medical support for pulmonary function, prophylactic antibiotics or immunoglobulins for recurrent infections, and an adapted treatment for malignancies [1, 2]. Unfortunately, a curative treatment for A-T is not yet available, and most patients with the classic form of the disease die before the age of 30 years.

During the past years, newborn screening (NBS) for severe combined Immunodeficiency (SCID) has become available and has been introduced in several countries [3]. Newborns with a SCID face the risk of life-threatening infections, caused by the very low numbers of T-cells. Early diagnosis is essential, as SCID patients treated with stem cell transplantation before the age of 3.5 months or before infections have occurred have a significant improved survival compared to those transplanted later or when infectious complications have accumulated [4, 5]. NBS for SCID is based on quantification of T-cell receptor excision circles (TRECs) in dried blood spots (Guthrie cards) of newborns. TRECs are formed during development of T-cells and are used as biomarker for the presence of T-cells. SCID patients do not have (functional) T-cells and therefore lack TRECs [6]. Low/absent TRECs can also be identified in neonates with T-cell impairment syndromes, newborns with T-cell impairment secondary to other neonatal conditions or patients with idiopathic lymphocytopenia [7-10]. Flow cytometry and genetic analysis are therefore required as confirmatory diagnostics to distinguish true SCID patients from these incidental findings.

It is known that part of the newborns with A-T have low TREC levels and therefore some (at that stage pre-symptomatic) A-T patients may be identified incidentally during NBS for SCID [11,12]. In April 2018, an implementation pilot study for NBS for SCID started in the Netherlands (SONNET-study, www.sonnetstudie.nl) [13]. In the Netherlands, screening for treatable disorders is undisputed, however, the discussion continues about screening for non-treatable disorders. Current guidelines of the Health Council of the Netherlands advise not to screen for non-treatable disorders and not to report (incidental) findings that may refer to untreatable disorders. However when there is potential health gain or prevention of health loss for a child with an early diagnosis,

this discussion can be re-opened in case there is supporting scientific evidence [14]. The fact that A-T should be regarded as untreatable, potentially leads to the unwanted situation of identifying a patient with A-T at a pre-symptomatic stage, based on NBS for SCID. From the experience of our national reference center for A-T at the Radboud University Medical Center, we know that the diagnostic process for A-T may take a long time and may include many procedures, e.g. lumbar punctures, muscle biopsies, and diagnostic x-rays (which are potentially harmful in the context of a DNA-repair disorder). Family members with a single *ATM* mutation have an increased risk for developing cancer (especially breast cancer in women) and cardiovascular diseases [15-17]. All the facets around the diagnosis of A-T combined, including the psychological stress, make it an interesting question whether parents of A-T patients would favor the possibility of an early diagnosis of A-T directly after birth, as a consequence (in fact an incidental finding) of SCID screening. In this study, a questionnaire was developed to investigate whether parents of a Dutch cohort of A-T patients would consider an early diagnosis beneficial or whether they would consider it harmful (taking away "the golden year(s)", i.e. the happy time before onset of symptoms). Now NBS for SCID is introduced in many other countries, this research contributes to the discussion whether A-T (or other untreatable disorders) should be diagnosed at a very early age when possible [18].

METHODS

Once every one or two years in the Netherlands, a national meeting with all A-T families is organized to give an update of recent developments in our clinic and in science, to discuss the progress in the medical literature, and simply to meet each other. During one of these meetings, parents and professionals discussed whether an early diagnosis of A-T would be advantageous. Based on this discussion, individual arguments were identified and processed into a questionnaire. Potential arguments were uncertainty up to the diagnosis, possible medical advantages, genetic counseling and family planning including potential prenatal diagnostics, loss of happy years, and early cancer screening for parents. To test the questionnaire, all doctors, nurses, and paramedics involved in the A-T team were sent a questionnaire. After this, the questionnaire was improved and sent to all Dutch parents of an A-T patient. Every household received two questionnaires (one for each parent). For every statement, a five-scale option was provided: strongly agree, agree, neutral, disagree, and strongly disagree. For the final question, three options were given: "the advantages outweigh the disadvantages," "the disadvantages out- weigh the advantages," or "I don't know." Parents were given the opportunity to motivate their definitive choice in an open box. The study was approved by the local medical ethical committee (METC 2018-4518).

RESULTS

In total, 64 A-T parents (32 families) received a questionnaire. The response rate was 55% as 35 A-T parents filled in the questionnaire. When parents filled in the questionnaire together, the questionnaire was counted twice. One grandmother filled in a questionnaire (instead of father); these data were included in the results. Fifteen A-T children had parents who both filled in a questionnaire, and five children had one parent who filled in the questionnaire. The cohort which replied to the questionnaire consisted of 21 classic A-T and 1 variant A-T (44 years old). The average age of alive classic A-T patients is 11 years (range 2 – 30), and five classic A-T patients deceased at an average age of 20 (range 14 – 26, 1 had missing data). The average age at diagnosis of A-T was 4.9 years old (range 1 – 10 years) for classic A-T. One variant A-T was diagnosed around 32 years old. No differences were observed between subgroups in this small and heterogeneous group of respondents.

Time to diagnosis

The first statement was aimed to verify whether parents would like to have known the diagnosis A-T shortly after birth in their specific situation. The majority (19/35) preferred hearing the diagnosis early (i.e., before start of symptoms) (Table 1, statement 1). There are multiple arguments that plea for an early diagnosis: uncertainty to diagnosis, early medical access, prenatal diagnostics, and cancer screening for parents (especially breast cancer screening for mothers). In accordance with expectations, many parents experience uncertainty towards the diagnosis A-T (31/35) (Table 1, statement 2). A parent illustrated the time before diagnosis: "We got the diagnosis A-T when our child was eight years old. The time before diagnosis was insecure. We questioned what the future of our daughter would look like. This period was difficult, sad and insecure." One of the other parents described the effect on their relationship: "The advantage of an early diagnosis to me is: the insecurity is taken away, you can get used to the feeling your child is ill and you can share this feeling with your partner. This leads to better binding between wife and husband. The sharing of grief prevents growing apart."

Medical access to a dedicated A-T referral center

A-T children (and adults) in the Netherlands are seen in our tertiary, national A-T referral center on an annual basis. The support in our center is provided by a large multidisciplinary team of doctors and paramedics. Some of the children receive (prophylactic) antibiotics or temporarily supporting immunoglobulins. However, as stated in the "Introduction" section, no treatment is available yet for A-T. Most parents see a medical advantage for their child with an early diagnosis (25/35) (Table 1, statement 3). Moreover, most parents point out the many diagnostic procedures and

its risks towards a diagnosis A-T: "My child had unnecessary diagnostic procedures. We could have taken action on time." Another parent: "An early diagnosis prevents burdensome or even dangerous diagnostic procedures (e.g., x-rays)." In other words, some parents had the feeling that their child had experienced unnecessary and preventable risks or damage.

Table 1. Results of the questionnaire of 35 A-T parents

	Statements	Strongly agree	Agree	Neutral	Disagree	Strongly disagree	Not filled in
1	In retrospect, we preferred hearing the diagnosis A-T in our case shortly after birth, although our child did not have symptoms at that time.	43% (15/35)	11% (4/35)	17% (6/35)	14% (5/35)	11% (4/35)	3% (1/35)
2	A diagnosis A-T based on the neonatal bloodspot screening prevents a period of uncertainty (start symptoms to eventual diagnosis). This time was a very uncertain period for me.	49% (17/35)	40% (14/35)	3% (1/35)	9% (3/35)	0% (0/35)	0% (0/35)
3	An early diagnosis gives my child early medical access. My child would have had an advantage to have that access.	51% (18/35)	20% (7/35)	6% (2/35)	20% (7/35)	3% (1/35)	0% (0/35)
4	An early diagnosis offers the opportunity to get access to genetic counseling for a potential child wish. For me, an early diagnosis is important for my future family planning.	51% (18/35)	31% (11/35)	9% (3/35)	6% (2/35)	0% (0/35)	3% (1/35)
5	An early diagnosis means that parents know they are carrier of a mutation in the *ATM* gene and with it an increased risk for cancer. It is an advantage to know this health risk. Therefore, an early diagnosis is important for my child.	37% (13/35)	26% (9/35)	20% (7/35)	9% (3/35)	6% (2/35)	3% (1/35)
6	An early diagnosis of A-T prevents parent to enjoy a healthy baby/ child in the first years of its life.	17% (6/35)	26% (9/35)	6% (2/35)	20% (7/35)	31% (11/35)	0% (0/35)

Family planning

Most parents see an early diagnosis of A-T as an important asset for future family planning (29/35) (Table 1, statement 4). For many, it is a decisive argument. Parents comment: "I want to make a conscious decision in my family planning" or "Whenever parents want more children, it is good to know there is a problem early on" or "The diagnosis in my first child took a long time. Meanwhile she got a little sister, who was 6 months old when the diagnosis A-T was made. The insecurity about her little sister was substantial. If I had known earlier, she would have been our only child. The risk is too high for me. Happily, her little sister is healthy."

Early screening for family members

The majority of parents see an early diagnosis of A-T as an advantage (21/35) (Table 1, statement 5) in order to be aware of their own health risk (increased risk for cancer). Importantly, in the Netherlands, female ATM mutation carriers have an adjusted screening program for breast cancer. A parent comments on this advantage: "It can be vital for an ATM mutation carrier to know to be at increased risk for cancer."

Happy years

The most important argument against an early diagnosis of A- T is the possible loss of happy years. Here, parents have differing opinions, 16 do agree that an early diagnosis would affect the first years of life and 18 do not (Table 1, statement 6). Parents who enjoyed the first healthy years stated: "I did not want to miss the first years of carefree enjoying my child." Another parent illustrated the fear that the diagnosis A-T brings: "In our case an early diagnosis would have led to more years of worries and fear. We had seven years without big worries, those are very precious to me." The ones who disagreed with the concept of happy years bring in the insecurity before diagnosis, illustrated in the earlier paragraph about time to diagnosis. One parent explained: "We had many worries and much insecurity. Our general practitioner and the regional hospital did not listen to us. I think we would enjoy this period more if we would have known the diagnosis."

Choice

Parents were asked to make a choice for or against the option for an early diagnosis of A-T in the asymptomatic phase. Twenty-six parents think the advantages outweigh the disadvantages, five parents think the disadvantages outweigh the advantages, and four parents do not know the answer to this question. Those who opposed against an early diagnosis were all parents of classic A-T patients (with differing ages). Afterwards, parents were asked what the decisive argument was in their definitive choice. Many of these arguments were discussed in the previous paragraphs: the proponents brought two main arguments to the fore, namely (1) the

uncertainty to diagnosis and its potential harmful diagnostic procedures and (2) the considerations in their family planning. The opponents emphasized the meaning of having a seemingly healthy child and with this the golden year(s).

The last question about the theoretical situation whether a test that could detect all patients with A-T via NBS should be implemented gave a similar result: 25 parents agreed, eight disagreed, and two did not fill in an answer.

DISCUSSION

After 92 years, since the discovery of A-T, our study seems to be the first study to explore the various concerns and anguishes A- T parents face before diagnosis and the impact of delayed diagnosis by answering a semi-structured questionnaire. NBS for SCID unexpectedly creates an opportunity for a very early A- T diagnosis. The benefits for SCID are clear: preventing severe infections by treatment with an early stem cell transplant, which improves life expectancy in this group [4]. Although this is a major argument to implement NBS for SCID, the benefits for the outcome of A-T patients are less clear. What started as a questionnaire to investigate parents' opinion about a current discussion in the Netherlands gave us an insight in parents' experience with A-T. Many parents had experienced uncertainty towards the diagnosis, having the feeling that their child is ill and that medical teams are not recognizing this. In these cases, the diagnosis meant a form of relief. Also, parents experienced that their child had been subjected to unnecessary potentially dangerous procedures, e.g., radiation as patients with A-T have an increased radiosensitivity. On the other hand, some parents who only had mild medical issues with their "clumsy" child had experienced the joy of seeing a (seemingly) healthy child grow up (also known as the "golden year(s)").

The knowledge of having a child with a genetic disease is important for parents. Many of them are young families with an ongoing child wish; others have a completed family, but would have decided otherwise if they knew the diagnosis A-T at that time.

In contrast to our expectations, A-T parents were divided about the loss of golden year(s). We expected that there was at least a loss of potential golden year(s) for all parents (also for those who favored an early diagnosis). In this questionnaire, many parents commented on the insecurity before diagnosis and the relief of the eventual diagnosis. These comments have emphasized the impact of not having a diagnosis. Also, parents confirm the importance that there is an advantage for heterozygous *ATM*

In the first statement, 19/35 parents would have preferred a diagnosis shortly after birth and 9/35 parents preferred not to hear the diagnosis at that time. As A-T is a devastating diagnosis that no parent ever wants to hear, it is most likely a negative memory which can influence a parent's opinion in this question. In the final question, we gave parents the opportunity to make a final choice taking all the advantages and disadvantages in their specific situation into consideration. The answer was based on parents' personal perspectives as parents could not decide for the entire group. We can imagine that parents would choose differently when they would have to make a decision for the whole group, instead of their specific personal situation, considering the complexity of this dilemma.

So far, limited studies show the percentages of A-T newborns that can be diagnosed as a result of NBS by retrospective analysis of NBS of Guthrie cards. At first a Californian group investigated the records of an A-T cohort over 25 years. Seven samples had low TREC levels in a cohort of 13 A-T patients (54%)[11]. In a small Swedish study, all four patients showed reduced numbers of TRECs. In the Dutch A-T cohort, we tested five Guthrie cards and four had low TRECs (unpublished data). Altogether, these data suggest that the majority of A-T patients will present with low TREC levels at birth and can possibly be diagnosed with A-T as a result of SCID screening. In the questionnaire presented to the A-T parents no exact numbers were mentioned as these are limited data.

A-T is a very rare disease with unique features. However, similar dilemmas about NBS have been discussed for other diseases, such as Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA). For these disorders, studies similar to the present one that we have performed for A-T showed a strong wish of parents (majority up to 95.9%) to implement DMD and SMA in neonatal screening programs without any therapeutic consequences at the time of the study [19]. In the 1980s, when the screening for DMD was first discussed, a similar retrospective study was performed to objectify parents' opinion on neonatal screening: a similar per- centage of parents (75%) was in favor of an early diagnosis, based on the same arguments as diagnostic delay, practical advantages, family planning, and emotional advantages [20]. In semi-structured interviews, parents reflected on the (delayed) diagnostic process and emphasized their feelings of worry and anxiousness of having an undiagnosed ill child and the eventual relief of being guided by a dedicated DMD team [21]. In all these aspects, our study shows similarities to the studies about screening for this untreatable disorder.

At this moment, a curative treatment for A-T is not available. NBS may identify pre-symptomatic patients, while -on the contrary- recent studies have also identified patients at the highest risk for early morbidity and mortality [22]. Whenever a form of

treatment becomes available, these groups at both ends of the clinical spectrum may be the first to benefit from early medical intervention. Undoubtedly, new technologic developments will influence the discussion about NBS for A-T.

This study has some limitations: it is a relatively short questionnaire in a small cohort. With only little number of cases no subgroup analysis was possible. Despite the limitations, however, we feel it is valuable to share the opinion of our cohort of A-T parents. Future research should address structured interviews with A-T parents. In addition, non-A-T parents should be introduced in this subject and asked for their opinion as well. This way, the current health policy regarding SCID screening in the Netherlands could be re-evaluated taking the A-T parents perspective into consideration.

CONCLUSION

The majority of parents of A-T patients would prefer to know the diagnosis A-T shortly after birth of their child, based on two major arguments: (1) the experienced insecurity in diagnostic trajectories and its impact on their lives and (2) the knowledge of being ATM mutation carriers when making decisions about family planning. Parents who opposed against an early diagnosis emphasized the joy of having a seemingly healthy child until diagnosis.

Acknowledgements
The authors thank all A-T families and members of the A-T expert team of the Radboud university medical center for their participation in this study, as well as the Twan Foundation for all fruitful collaborations. The study was supported by the Action for A-T foundation (project 4519) and ZonMW (SONNET study, project 543002002).

Author's contributions
MS, CW, MvdB, MW designed the research; MS, MB and MdV developed the questionnaire; MS collected and analyzed the data; MS and MB wrote the paper; MvdB, MW, CMR edited the paper; all authors approved the final version.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

The study was supported by the Action for A-T foundation (project 4519) and ZonMW (SONNET study, project 543002002).

Ethical approval

This article includes a questionnaire study that is not subjected to the Medical Research Involving Human Subjects Act (WMO). The questionnaires were in accordance with the ethical standards of the institutional ethics committee (METC 2018-4518).

Informed consent

Participation in the questionnaire after receiving the invitation implied consent. Informed consent was obtained from all individual participants included in the study.

REFERENCES

1. Rothblum-Oviatt C, Wright J, Lefton-Greif MA, McGrath-Morrow SA, Crawford TO, Lederman HM (2016) Ataxia telangiectasia: a review. Orphanet J Rare Dis 11:159
2. Schoenaker MH, Suarez F, Szczepanski T, Mahlaoui N, Loeffen JL (2016) Treatment of acute leukemia in children with ataxia telangiectasia (A-T). Eur J Med Genet 59:641-646
3. King JR, Hammarstrom L (2018) Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. Journal of clinical immunology 38:56-66
4. Heimall J, Cowan MJ (2017) Long term outcomes of severe combined immunodeficiency: therapy implications. Expert review of clinical immunology 13:1029-1040
5. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, Kapoor N, et al. (2014) Transplantation outcomes for severe combined immunodeficiency, 2000-2009. The New England journal of medicine 371:434-446
6. Chan K, Puck JM (2005) Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol 115:391-398
7. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, Baker M, et al. (2014) Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama 312:729-738
8. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterstrom RH, King J, Winiarski J, von Dobeln U, Hammarstrom L (2017) Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Journal of clinical immunology 37:51-60
9. Audrain MAP, Leger AJC, Hemont CAF, Mirallie SM, Cheillan D, Rimbert MGM, Le Thuaut AM, Sebille-Rivain VA, Prat A, Pinel EMQ, Divry E, Dert CGL, Fournier MAG, Thomas CJC (2018) Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). Journal of clinical immunology 38:778-786
10. Thomas C, Durand-Zaleski I, Frenkiel J, Mirallie S, Leger A, Cheillan D, Picard C, Mahlaoui N, Riche VP, Roussey M, Sebille V, Rabetrano H, Dert C, Fischer A, Audrain M (2019) Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clinical immunology (Orlando, Fla) 202:33-39
11. Mallott J, Kwan A, Church J, Gonzalez-Espinosa D, Lorey F, Tang LF, Sunderam U, Rana S, Srinivasan R, Brenner SE, Puck J (2013) Newborn screening for SCID identifies patients with ataxia telangiectasia. Journal of clinical immunology 33:540-549
12. Borte S, von Dobeln U, Fasth A, Wang N, Janzi M, Winiarski J, Sack U, Pan-Hammarstrom Q, Borte M, Hammarstrom L (2012) Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood 119:2552-2555
13. Blom M, Pico-Knijnenburg I, Sijne-van Veen M, Boelen A, Bredius RGM, van der Burg M, Schielen P (2017) An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. Clinical immunology (Orlando, Fla) 180:106-110
14. Gezondheidsraad (2015) Neonatale screening: aanbevelingen.
15. Renault AL, Mebirouk N, Cavaciuti E, Le Gal D, Lecarpentier J, d'Enghien CD, Lauge A, et al. (2017) Telomere length, ATM mutation status and cancer risk in Ataxia-Telangiectasia families. Carcinogenesis 38:994-1003

16. van Os NJ, Roeleveld N, Weemaes CM, Jongmans MC, Janssens GO, Taylor AM, Hoogerbrugge N, Willemsen MA (2016) Health risks for ataxia-telangiectasia mutated heterozygotes: a systematic review, meta-analysis and evidence-based guideline. Clinical genetics 90:105-117
17. Jerzak KJ, Mancuso T, Eisen A (2018) Ataxia-telangiectasia gene (ATM) mutation heterozygosity in breast cancer: a narrative review. Current oncology (Toronto, Ont) 25:e176-e180
18. Kwan A, Puck JM (2015) History and current status of newborn screening for severe combined immunodeficiency. Seminars in perinatology 39:194-205
19. Wood MF, Hughes SC, Hache LP, Naylor EW, Abdel-Hamid HZ, Barmada MM, Dobrowolski SF, Stickler DE, Clemens PR (2014) Parental attitudes toward newborn screening for Duchenne/Becker muscular dystrophy and spinal muscular atrophy. Muscle Nerve 49:822-828
20. Firth MA, Wilkinson EJ (1983) Screening the newborn for Duchenne muscular dystrophy: parents' views. Br Med J (Clin Res Ed) 286:1933-1934
21. Bendixen RM, Houtrow A (2017) Parental Reflections on the Diagnostic Process for Duchenne Muscular Dystrophy: A Qualitative Study. J Pediatr Health Care 31:285-292
22. van Os NJH, Jansen AFM, van Deuren M, Haraldsson A, van Driel NTM, Etzioni A, van der Flier M, Haaxma CA, Morio T, Rawat A, Schoenaker MHD, Soresina A, Taylor AMR, van de Warrenburg BPC, Weemaes CMR, Roeleveld N, Willemsen M (2017) Ataxia-telangiectasia: Immunodeficiency and survival. Clinical immunology (Orlando, Fla) 178:45-55

SUPPLEMENTARY MATERIAL

Questionnaire for parents of a child with Ataxia Telangiectasia (A-T)

Newborns in the Netherlands are tested for a number of rare, serious and treatable diseases via the newborn screening program. Timely detection and treatment of these diseases will prevent or limit serious health damage. In 2017, the State Secretary for Health, Welfare and Sport decided to expand the Dutch newborn screening program with twelve new diseases. One of these new diseases is Severe Combined Immunodeficiency (SCID), a rare, serious disease of the immune system that can be cured with stem cell transplantation. In the newborn screening test for SCID, certain markers of the immune system are low or absent at birth. These markers can also be low or absent in A-T patients. As a result, children with A-T may have an abnormal newborn screening result, even though there are no symptoms of A-T in the neonatal period. We wonder what the advantages and disadvantages are of an early diagnosis A-T. We believe that the experience and opinion of A-T parents are important aspects in this discussion. We would therefore like to present a number of statements to gain more insight into the considerations regarding an early diagnosis of A-T, from the perspective of parents.

Father's name:
Mother's name:
Name and date of birth child:

We would prefer it if both parents filled in a questionnaire separately.

This form was filled in by:

Father Mother Father and mother together

Statements

After each statement, you can choose between five possibilities: strongly agree – agree-neutral – disagree – strongly disagree. Please encircle the answer that best applies to you.

Statement. In retrospect, we would rather have heard the diagnosis A-T of our child shortly after birth, even though our child had no symptoms of the disease at that time.

Your opinion:

1. Strongly agree. 2. Agree. 3. Neutral. 4. Disagree. 5. Strongly disagree

Various considerations could play a role in the answer to the above question. We would like to know your opinion about early diagnostics (shortly after birth). Could you therefore indicate to what extent you agree with the following statements?

Statement. A diagnosis A-T based on newborn screening prevents a period of uncertainty (from start of symptoms to final diagnosis).

Your opinion:
There was a time where symptoms of A-T were present, but there was no diagnosis yet. This was a very uncertain period for me.

1. Strongly agree. *2. Agree.* *3. Neutral.* *4. Disagree.* *5. Strongly disagree*

Statement. An early diagnosis of A-T gives my child early medical access. Therefore, supportive therapy can be started in an early phase of the disease.

Your opinion:
My child would have had an advantage to have that early medical access.

1. Strongly agree. *2. Agree.* *3. Neutral.* *4. Disagree.* *5. Strongly disagree*

Statement. An early diagnosis of A-T offers the opportunity to get access to genetic counselling.

Your opinion:
For me, an early diagnosis is important for my future family planning.

1. Strongly agree. *2. Agree.* *3. Neutral.* *4. Disagree.* *5. Strongly disagree*

Statement. An early diagnosis of A-T deprives parents of the opportunity to enjoy a (seemingly) healthy newborn/child in the first years of life.

Your opinion:
I would have enjoyed my child's early childhood less if I had known the diagnosis A-T shortly after birth.

1. Strongly agree. *2. Agree.* *3. Neutral.* *4. Disagree.* *5. Strongly disagree*

Statement. An early diagnosis of A-T means that parents will also know that they are carriers of a mutation in the *ATM* gene with an (mildly) increased risk of developing cancer.

Your opinion
It is an advantage to be aware of the above-mentioned health risk for parents (and other family members). That is why an early diagnosis is important for both me and my child.

Space for any additional comments or suggestions:

Do you have arguments to be for or against an early diagnosis of A-T, based on newborn screening in the first week of life, other than described in the above statements? If yes, please fill in below:

For an early A-T diagnosis	**Against an early A-T diagnosis**

Choice
After you have considered all the above questions and statements, we would like to ask you what you think about the introduction of a new technique in the newborn screening program that leads to an early diagnosis of A-T (in the first weeks of life) in newborns in the Netherlands.

The following would apply to me:
1. I am FOR an early diagnosis: the benefits outweigh the disadvantages.
2. I am AGAINST an early diagnosis: the disadvantages outweigh the benefits.
3. I don't know

What is the decisive argument in your consideration?

Other questions

Finally, we have two statements to gain insight into the possible follow-up procedure that we should follow with abnormal screening results.

Statement. With the current technique, A-T is "accidentally" detected as an incidental finding of newborn screening for SCID. However, the screening is intended to diagnose SCID (see above). In the case of an abnormal SCID screening result that turns out not be SCID after follow-up diagnostics, the newborn could have A-T. In this case, a medical team should keep a close eye on the patient, but diagnostics for A-T should not be applied. Additional diagnostics for A-T should only be used if symptoms of A-T begin to occur

Your opinion:

1. Strongly agree. *2. Agree.* *3. Neutral.* *4. Disagree.* *5. Strongly disagree*

Statement. If another technique was available that would be able detect all children with A-T with newborn screening, A-T should be included in the regular Dutch newborn screening program.

Your opinion:

1. Agree, the benefits outweigh the disadvantages.
2. Disagree, the disadvantages outweigh the benefits.

Thank you for completing this questionnaire.

Space for any additional comments or suggestions:

CHAPTER 8

Dilemma of reporting incidental findings in newborn screening programs for SCID: parents' perspective on ataxia telangiectasia

Maartje Blom, Michiel H. D. Schoenaker, Myrthe Hulst, Martine C. de Vries, Corry M. R. Weemaes, Michèl A. A. P. Willemsen, Lidewij Henneman and Mirjam van der Burg

Frontiers in Immunology, 2019; 10: 2438

ABSTRACT

Background

Ataxia Telangiectasia (A-T) is a severe DNA repair disorder that leads to a broad range of symptoms including neurodegeneration and a variable immunodeficiency. A-T is one of the incidental findings that accompanies newborn screening for severe combined immunodeficiency (SCID), leading to an early diagnosis of A-T at birth in a pre-symptomatic stage. While some countries embrace all incidental findings, the current policy in the Netherlands on reporting untreatable incidental findings is more conservative. We present parents' perspectives and considerations on the various advantages versus disadvantages of early and late diagnosis of A-T.

Methods

A questionnaire was developed and sent to 4000 parents of healthy newborns who participated in the Dutch SONNET-study (implementation pilot for newborn screening for SCID). The questionnaire consisted of open-ended and scale questions on advantages and disadvantages of early and late diagnosis of A-T. To address potential bias, demographic characteristics of the study sample were compared to a reference population.

Results

A total of 664 of 4000 parents sent back the questionnaire (response rate 16.6%). The vast majority of parents (81.9%) favored early diagnosis of A-T over late diagnosis. Main arguments were to avoid a long period of uncertainty prior to diagnosis and to ensure the most optimal clinical care and guidance from the onset of symptoms. Parents who favored late diagnosis of A-T stated that early diagnosis would not lead to improved quality of life and preferred to enjoy the so-called 'golden years' with their child. The majority of parents (81.1%) stated that they would participate in newborn screening for A-T if a test was available.

Conclusions

Reporting untreatable incidental findings remains a disputed topic worldwide. Although the current policy in the Netherlands is not to report untreatable incidental findings, unless the health advantage is clear, the majority of parents of healthy newborns are in favor of an early A-T diagnosis in the pre-symptomatic phase of the disorder. Our results as well as other studies that showed support for the screening of untreatable disorders may serve as valuable tools to inform policymakers in their considerations about NBS for untreatable disorders.

INTRODUCTION

In the last years, newborn bloodspot screening (NBS) for severe combined immunodeficiency (SCID) has been introduced in several screening programs worldwide [1-3]. NBS for SCID is based on the detection of T-cell receptor excision circles (TRECs) in dried blood spots. TRECs are formed during the T-cell receptor rearrangement, therefore serving as a biomarker for newly formed T-lymphocytes. SCID patients do not have (functional) T-cells and therefore lack TRECs [4]. Several studies have shown that NBS for SCID is accompanied by a high number of incidental findings. Low/absent TRECs can also be identified in neonates with T-cell impairment syndromes (such as DiGeorge Syndrome, Down Syndrome or Ataxia Telangiectasia), newborns with T-cell impairment secondary to other neonatal conditions or patients with idiopathic lymphocytopenia [3, 5, 6]. The relatively high number of incidental findings is met with hesitations by policy makers responsible for making decisions with regard to implementation of SCID in NBS programs. However, these infants with non-SCID lymphopenia disorders do seem to benefit from early detection and treatment, for example by the prevention and reduction of infections by antibiotic prophylaxes and protective measures [7]. In addition, possible harm by receiving life attenuated rotavirus or BCG vaccines can be avoided [8]. There are however, untreatable conditions with low TRECs that present asymptomatic at birth and for which health benefits by early detection remain disputable. A key example of these untreatable conditions is Ataxia Telangiectasia (A-T).

A-T is a rare, autosomal recessively inherited disorder caused by mutations in the *Ataxia Telangiectasia Mutated* (*ATM*) gene. This DNA repair disorder leads to a combination of systemic and neurological symptoms, including progressive ataxia, ocular telangiectasias, predisposition to malignancies and a variable immunodeficiency [9]. Patients with classic A-T are asymptomatic in the first year of life, but progressive symptoms will develop shortly after. The prevalence of A-T is estimated to be between 1 in 40,000 and 1 in 100,000 live births [9]. A-T is a complex disease to diagnose as clinical presentation and/or laboratory findings vary between patients. A curative treatment for A-T is not yet available, and most patients with the classic form of the disease die before the age of 30 years [9]. Optimal symptomatic treatment in the setting of a dedicated and experienced multidisciplinary team of health care professionals is of great importance [10]. Of note, heterozygous carriers of a pathogenic *ATM* mutation, i.e. the parents of the newborn that underwent NBS, have a slightly decreased life expectancy and increased risk of developing cancer, especially breast cancer [11, 12]. This implies that NBS for SCID might reveal a health risk for family members of the screened newborn in addition to risks for the newborn itself.

A-T was first described as an incidental finding to NBS for SCID in 2013 in California [13]. Retrospective analysis of NBS cards of A-T patients showed that not all A-T patients present with low TRECs at birth [13, 14]. However, no significant associations could be identified between the newborn TREC numbers and phenotypic clinical and laboratory features of A-T (such as age at presentation with neurological symptoms, total $CD3^+$ T- cell counts or time between symptom-onset and diagnosis). Since then, multiple NBS programs with different assays and cut-off values have identified A-T patients based on low TRECs over the last few years (California N = 5 [1], France N = 1 [5], Sweden N = 1 [6]).

NBS for SCID based on TREC-quantification is intended to identify SCID patients at birth in order to enable early diagnosis and treatment of an otherwise fatal disorder. Conventional follow-up diagnostics after abnormal TREC results consist of flow cytometry and genetic confirmation of the underlying mutation. By adding the *ATM* gene in follow-up gene panels, NBS programs engage in an active search for A-T patients with the additional chance of identifying carriers of *ATM* mutations. While the reporting of clinically relevant and treatable (incidental) disorders is undisputed in the field of (neonatal) screening, the current policy on reporting untreatable (incidental) disorders remains controversial. The Wilson and Jungner screening criteria (1968) guide towards screening for treatable disorders. In addition, the Health Council of the Netherlands states that NBS for untreatable disorders and reporting of untreatable incidental findings would not be in the immediate health interest of the child [15]. With these considerations in mind and based on expert opinions that question the added value of early diagnosis in A-T patients, Dutch experts decided to exclude the *ATM* gene from the NBS follow-up gene panel. There were, however, two major conditions in this follow-up produce in the interest of potential A-T patients. First, in the case of low TRECs/T-cells without a confirmed underlying genetic defect but with an indication for hematopoietic stem cell transplantation (HSCT), *ATM* mutations have to be ruled out before starting with conditioning regimes. Second, in the case of idiopathic T-cell lymphocytopenia (without genetic diagnosis) and no indication for HSCT, the newborn will be enrolled in out-patient clinical follow-up visits. If any clinical symptoms matching A-T start to occur, additional diagnostics (*ATM* gene analysis) will be initiated immediately. This follow-up protocol ensures that during the Dutch implementation pilot for NBS for SCID (SONNET-study, www.sonnetstudie.nl) untreatable incidental findings will not be reported and A-T will not be an incidental finding to NBS for SCID in the Netherlands.

The perspective of parents as key stakeholders in NBS is of great value in policymaking. The aim of this study is therefore to gain insight into parents' perspectives about the early detection of A-T. Empirical data on the views of parents on early detection of A-T will provide insight into the public acceptance of untreatable incidental findings to NBS.

METHODS

Study population and procedure

The study encompasses a cross-sectional survey study amongst parents of healthy newborns. A questionnaire was sent to 4000 Dutch parents of healthy newborns. Only parents from the pilot-provinces Utrecht, Gelderland and Zuid-Holland who participated in the SONNET-study were invited to participate (www.sonnetstudie.nl). In order to participate in the SONNET-study, parents have to express verbal consent when the heel prick is performed. If parents object to the SONNET-study and with that NBS for SCID, this was noted on the blood spot card and registered in the screening laboratories. Parents who objected to participation in the SONNET-study or the entire NBS program were not invited for this survey study. The questionnaire focused on a potential incidental finding of NBS for SCID, therefore parents were approached eight to ten weeks after their child received the heel prick in the hope information about NBS could still be recalled. Questionnaires could not be sent out earlier as parents with abnormal screening results for their latest child were excluded from the study, and it can take up to five weeks to process NBS results from all disorders of the entire program. If the newborn deceased in this period after birth, parents were not invited to participate. Parents' addresses were obtained via the National Institute for Public Health and the Environment (RIVM) after approval of working party Management Information System (MIS) of the RIVM. Parents were able to send back a printed questionnaire or to fill in the questionnaire online by following a link or scanning a QR-code. The survey was available in Dutch and accompanied by a cover letter from the RIVM with information about the study and privacy regulations. Filling out the questionnaire was voluntary and participation after receiving the invitation implied consent. All data was analyzed anonymously. Due to privacy reasons, no reminders were allowed to be sent. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, the Netherlands (MEC-2017-1146).

Questionnaire design and measures

A questionnaire about A-T was specifically developed for this study by a multidisciplinary group of experts on A-T, NBS, medical ethics and survey studies. The questionnaire was based on the literature and questionnaires previously used for investigating parents' perspectives on NBS e.g. for Pompe disease [16]. The questionnaire focused on the dilemma of early diagnosis of A-T and consisted of open questions with additionally multiple choices, scales and yes/no answers. Since the disorder A-T is rare and parents are not acquainted with the symptoms and course of the disorder, the questionnaire started with a background information section on A-T (Supplementary Section A). The questionnaire consisted of four sections (Supplementary Sections

B-E): 1) scenarios about early/late diagnosis of A-T, 2) statements about advantages and disadvantages of early diagnosis A-T, 3) final questions with decisive arguments and 4) sociodemographic questions. A small test phase was conducted to check for concept and wording of questions. The questionnaire has 23 questions in total and took approximately twenty minutes to complete.

The scenarios included two cases of children with A-T: one with a late diagnosis of A-T at the age of four years and one with an early diagnosis of A-T at birth as a result of NBS for SCID (Supplementary Section B). Parents were asked to list the advantages and disadvantages of both scenarios from their perspective in a free text response. The open questions were analyzed by dividing the answers into categories using a dichotomous variable scoring system. Answers could be assigned to multiple categories. Open questions were categorized independently by two different researchers to enhance the internal validity (MB and MH).

The scenarios were followed by eleven statements about advantages of early detection and nine statements about disadvantages of early detection of A-T (Supplementary Section C). Parents could indicate their degree of support on a five-point Likert scale (1 = totally disagree to 5 = totally agree). Two statements were added to the questionnaire that were also included in the study of Schoenaker et al. (2020) that aimed to investigate the perspective of A-T families on early detection of A-T. This way, a comparison could be made to the perspective of parents of A-T patients. Parents were additionally asked to indicate their degree of support on a five point scale (1 = totally disagree to 5 = totally agree) about the current follow-up policy after an abnormal NBS result for SCID. The statements included "In the case of an abnormal SCID screening result, diagnostics for A-T should be applied immediately" and "In the case of an abnormal SCID screening result that turns out not be SCID after follow-up diagnostics, diagnostics for A-T should not be applied. Additional diagnostics for A-T should only be used if symptoms of A-T begin to occur".

The final questions included two hypothetical questions (Supplementary Section D). The first question "If a test would be available to screen all newborns for A-T, would you personally participate in this screening?" had a five point scale answer (1 = yes, 2 = probably yes, 3 = don't know, 4 = probably no, 5 = no) . Parents were asked to choose their decisive arguments from multiple answers. The decisive argument to use or not use a hypothetical screening test for A-T was considered valid only if the respondent had a matching yes/probably yes or no/probably no answer. If the respondent noted more than one decisive argument, the answer was coded as 'other'. The second question "Do you think A-T should be included in the NBS program?" could be answered on a three point scale (1 = yes, 2 = don't know, 3 = no).

The questionnaire ended with a sociodemographic section that included questions about gender, age, ethnicity and educational level. Respondents were asked to indicate the highest level of education they had completed. Education level was grouped into three categories: low, middle and high (Table 1). Ethnicity was coded as 'Dutch' or 'Other' based on the country of birth and country of birth of mother and father. Due to underrepresentation of the non-Dutch group, no distinction was made between Western and non-Western background. Furthermore, parents were asked to fill in the number of children they have/had, including their age, NBS results and health status. Civil registry status 'single' and NBS parameters 'not participated' and 'abnormal screening results' were strongly underreported in the study population, therefore the relationship between variables and attitude towards early detection A-T was not analyzed.

Statistical analysis

Statistical analysis was carried with SPSS version 25.0 for Windows (SPSS, Inc., Chicago, IL, USA). Sociodemographic characteristics of participants were compared to the Dutch reference population reported by Statistics Netherlands with one sample-t-test for age, chi square test for trend for ordered categories and Pearson's chi square test for other characteristics. Descriptive statistics were used to describe characteristics of the respondents. Descriptive statistics were additionally used to determine frequencies of answers of participants categorized as dichotomous variables. Ordinal variables from scaled items are reported as means. Missing data in the study did not exceed 5% in any measure. For multivariate logistic regression analyses, items consisting of five-point scales were summarized to three-point scales: 1 = (totally) disagree, 2 = do not disagree/do not agree and 3 = (totally) agree. Multivariate logistic regression analysis was performed to determine whether the variables, age, gender, ethnicity, educational level were associated with the "if a test was available to screen all newborns for A-T, I would participate' and 'if a test was available, A-T should be added to newborn screening program'. Having one child, having a child with a (genetic) condition and having a family member with a hereditary disorder were included as variables as well. Standardized regression coefficients (β) are reported as an expression of the strength of the associated variables. Missing data were not analyzed in regression analyses. P-values < 0.05 were considered statistically significant.

Table 1. Sociodemographics of the respondents

Variables	Research population N = 659	Reference group Dutch population N (x1000)	P-value
Age in years (SD)			
Mean age of mothers in research/reference population	34.7 (4.81)	34.2	0.341
Mean age of fathers in research/reference population	32.1 (4.22)	31.3	<0.001
Gender, N (%)		Dutch population age 20-50 years[b]	<0.001
Male	86 (13.1)	3 304 (50.3)	
Female	571 (86.9)	3 266 (49.7)	
Missing	2		
Ethnicity, N (%)		Dutch population age 20-50 years[c]	<0.001
Dutch	569 (86.9)	4 675 (70.6)	
Other	86 (13.1)	1 932 (29.4)	
Missing	4		
Civil registry, N (%)		Dutch parents[d]	<0.001
Single	19 (2.9)	572 (21.6)	
Living together/married	637 (97.1)	2 024 (78.4)	
Missing	3		
Highest education level, N (%)[e]		Dutch population age 25-45 years[e]	<0.001
Low	24 (3.7)	585 (30.9)	
Middle	143 (21.8)	1643 (38.1)	
High	490 (74.6)	1908 (29.4)	
Missing	2		
Number of children, N(%)		Dutch parents[f]	0.0149
1	324 (49.5)	71.9 (44.2)	
2	219 (33.5)	62.5 (38.5)	
≥3	111 (17.0)	28.1 (17.3)	
Missing	5		

Table 1. Continued

Missing values were excluded from the percentages.

a. Reference population Dutch Parents [30]. One sample T-test.
b. Reference population Dutch population age 20-50 years [17]. $\chi 2$ test
c. Reference population Dutch population age 20-50 years [17]. $\chi 2$ test
d. Reference population Dutch population households ([19]). $\chi 2$ test
e. Low: primary education, lower vocational education, lower and middle general secondary education
   Middle: middle vocational education, higher secondary education, and pre-university education
   High: higher vocational education and university.
   Reference population Dutch population age 25-45 years [18]. $\chi 2$ test
f. Reference population Dutch parents [19]. $\chi 2$ test for trend.

RESULTS

Response and demographics

A total of 664 of 4000 parents sent back the questionnaire leading to a response rate of 16.6%. The majority of parents responded by sending the printed questionnaire back (N = 550/82.8%) compared to 114 (17.2%) parents who filled in the questionnaire online. Questionnaires where at least the statements about disadvantages and advantages of early diagnose A-T were completed (Supplementary Section C), were considered eligible for analysis. Based on this criterion, five questionnaires were excluded from the study resulting in the analysis of 659 questionnaires.

The respondents' characteristics are given in Table 1. The mean age of respondents was 32.4 years (range 20 - 47 years). Women were overrepresented in the respondent group (86.9%). Compared to the reference population, the respondents were more highly educated and more likely to have a Dutch ethnic background [17, 18]. The average number of children was 1.73 (range 1 – 11 children) compared to 1.61 in the reference population of Dutch parents [19] (Table 1). The vast majority of parents (99.5%) indicated that all their children had participated in the Dutch NBS program. Of the five parents that indicated that one of their children had not participated, all stated that newborn screening was performed abroad. As expected, parents reported that most NBS results were normal. Abnormal results included congenital hypothyroidism (N = 1) and carrier status of sickle cell anemia (N = 1). Twenty-four parents with a child with a (genetic) condition mentioned a range of hereditary disorders whereas participations who indicated the presence of a family member with a hereditary disorder (17.2%) mentioned a broad spectrum of as well disorders (Table 2).

Table 2. Participation NBS, health status of the children and familial hereditary disorders

	Research population N = 659	**%**
Did all your children participate in the Dutch NBS program?		
Yes	644	99.5
No	5	0.5
Missing	10	
What was the NBS result for your child(-ren)?		
Normal	643	99.5
Abnormal	2	0.3
I'd rather not say	1	0.2
Missing	13	
Are your children healthy? [a]		
Yes	628	96.0
No	24	3.7
I'd rather not say	2	0.3
Missing	5	
Do you have a family member with a hereditary disorder? [b]		
Yes	112	17.2
No	501	76.8
I don't know	35	5.4
I'd rather not say	4	0.6
Missing	7	

Missing values were excluded from the percentages. [a] Answers included a wide variety of hereditary disorders including Down Syndrome, Fragile X-syndrome, metabolic diseases and diabetes mellitus type 1. [b] Answer included a broad spectrum of disorders such as malignancies, diabetes mellitus, cardiovascular diseases and autoimmune diseases.

Attitude towards late and early detection of A-T

In total, 652 out of 659 parents listed advantages and disadvantages to the scenarios about late and early detection of A-T (Table 3). The majority of parents (57.1%) indicated the 'golden/happy' years, the asymptomatic years without worries or anxiety, as the main advantage of late diagnosis of A-T. In addition, parents mentioned that it would be an advantage for the child to not receive medical labeling from birth, allowing them to develop at their own pace. Other advantages mentioned were: the opportunity to fully enjoy the maternity period (10.3%) and the ability to have another child without any worries

about the disease (8.5%). Even though parents were asked to indicate the advantages of late detection, more than a quarter of parents stated that they did not see any advantages of late detection of A-T (26.1%). The main disadvantage of late detection of A-T in the perspective of parents was linked to the hereditary character of the disorder (46.2%). The case described the situation in which the couple already had a second child when their first child was diagnosed with A-T. Not being able to make a well-informed decision about family planning or prenatal diagnostics was an important negative aspect for parents. Parents also associated late diagnosis of A-T with a delayed start of medical access (guidance and surveillance of the patient and family) (42.6%) a long period of uncertainty and worries (30.8% and 21.5%) and delayed breast cancer screening for the mother of the A-T patient (18.4%). One eighth of the parents (12.8%) additionally mentioned not being able to mentally or financially prepare for the diagnosis as a disadvantage.

Table 3. Advantages and disadvantages of late and early detection of A-T according to parents (N = 652 respondents)

Late detection A-T		**Early detection A-T**	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Carefree period (57.1%)	Heredity (chance of another child with A-T) (46.2%)	Start with supportive treatment (49.2%)	No worry-free period (48.9%)
Parents who stated they saw no advantages in late detection of A-T (26.1%)	Delayed start of treatment/ surveillance (42.6%)	Clarity, knowing what to expect (35.6%)	Unable to enjoy the maternity period (47%)
No medical labeling of child (11.2%)	Long period of uncertainty (30.8%)	Surveillance by specialists (37.7%)	The baby has no symptoms yet (23.2%)
Being able to fully enjoy the maternity period (10.3%)	Long period of worries (21.5%)	Early breast cancer screening mother (27.2%)	Devastating news in a mentally emotional period (15.1%)
Being able to make a carefree choice to have another child (8.5%)	Delayed breast cancer screening mother (18.4%)	Being able to prepare (mentally/ practically) for a sick child (26.3%)	Insecurity about the future (14.3%)
	No time to prepare (mentally/ practically)/ make adjustments in your life (12.8%)	Being able to make informed reproductive choices (13.1%)	Difficulty to process information directly after birth (8.7%)

The main advantage of early detection of A-T from a parents' perspective was the ability to start with supportive treatment (e.g. physiotherapy) and receiving the most optimal clinical guidance right from the start (49.2%). Surveillance by a multidisciplinary team of specialists was mentioned by 37.7% of the parents as well. Parents highly valued clarity and knowing what to expect in contrast to the uncertainty and insecurity that are accompanied by a late diagnosis of A-T. Other advantages mentioned were: early breast cancer screening for the mother of the A-T patient (27.2%), the ability to (mentally and practically) prepare for a life with a child with a serious condition (26.3%) and the opportunity to make an informed reproductive choice (13.1%). The exclusion of a worry- or care-free period (48.9%) next to the inability to enjoy the maternity period (47%) were listed by parents as the main disadvantages of early detection of A-T. These disadvantages were directly linked to the difficulty to process such devastating news in an emotional and hormonal period after birth (15.1% and 8.7%). Other disadvantages of early detection of A-T mentioned were: the asymptomatic newborn ("the baby has no symptoms yet") (23.2%) and the insecurity with regard to the future (14.3%). In general, parents were able to indicate more advantages for early detection than for late detection of A-T. Several parents mentioned the difficulty of the dilemma and the ability to argue for both sides.

Level of agreement with regard to advantages and disadvantages early diagnosis A-T
Parents were asked to indicate their level of agreement of support for eleven statements about advantages of early detection and nine statements about disadvantages of early detection of A-T. The statement with the highest level of support indicated that parents value the fact that an early diagnosis of A-T will ensure that a child with A-T will immediately receive optimal guidance when the first symptoms occur (rating mean 4.5) (Table 4). Additionally, most parents agreed that early diagnosis of A-T would prevent a long period between the first symptoms and eventual diagnosis (rating mean 4.2) and with that, a long time of uncertainty for parents (rating mean 4.2). Family planning, early breast cancer screening for mothers and the opportunity to make adjustments into your lives were all advantages of early diagnosis A-T parents agreed with. In contrast, saving extra health associated costs and the idea that parents will be able to take better care of their child if diagnosed early, do not show the same levels of support (both rating mean 3.3). Parents perceive the most important disadvantages of early detection of A-T as 'early detection of A-T overburdens parents with information about an untreatable disease during the maternity period' and 'early detection of A-T deprives parents of the opportunity to enjoy a seemingly healthy baby in the first months/years of life (both rating mean 3.4) (Table 5). Other disadvantages were met with neutrality or disagreement. For most parents, the fact that A-T cannot be cured or treated is not perceived as a

disadvantage of early detection (rating mean 2.5). Arguments as 'taking life as it comes' (rating mean 2.5) or 'early detection will reduce the bond between parents and child (rating mean 2.5) were not agreed with. Several parents mentioned that they agreed with the statements about late detection of A-T, but that they see more benefits in early detection of A-T.

Table 4. Level of agreement with regard to advantages of early detection of A-T

Survey question:	**Level of agreement [a] (%)**					**Rating mean (SD)**
	Fully disagree				**Fully agree**	
Early detection of A-T ensures that a child with A-T can immediately receive optimal guidance when the first symptoms occur	1.7	1.6	0.8	34.3	61.6	4.5 (0.75)
Early detection of A-T prevents a long period between the first symptoms and the eventual diagnosis	1.9	3.7	7.9	47.0	39.4	4.2 (0.87)
Early detection of A-T provides parents with the opportunity to make informed choices about family planning	2.8	3.3	5.8	43.1	45.0	4.2 (0.91)
Early detection of A-T prevents a long period of uncertainty for parents	3.1	5.3	6.9	40.1	44.2	4.2 (0.99)
Early detection enables parents to make early adjustments into their lives (for example wheelchair accessible house)	2.0	6.2	13.1	49.9	28.1	4.0 (0.92)
It is an advantage that parents are informed about the slightly increased risk of developing breast cancer for the mother	2.5	5.0	12.3	45.2	34.2	4.0 (0.95)
Early detection of A-T ensures that parents can adjust their expectations about the condition of their child	2.3	7.3	10.0	50.9	29.0	4.0 (0.95)
Early detection of A-T prevents unnecessary additional tests	1.9	7.8	14.2	51.5	24.3	3.9 (0.93)
Early detection of A-T prevents multiple visits to the hospital	2.8	15.6	20.3	42.5	20.3	3.6 (1.05)
Early detection of A-T saves extra health costs	6.1	17.9	26.4	36.3	12.6	3.3 (1.10)
Early detection of A-T ensures that parent can take better care of their child	10.0	17.2	25.6	27.6	19.0	3.3 (1.24)

SD = Standard deviation. [a] Five-point rating scale: 1 = fully disagree; 5 = fully agree; N = 659 respondents. Missing values are excluded from the percentages.

Table 5. Level of agreement with regard to disadvantages of early detection of A-T

Survey question:	Level of agreement [a] (%)					Rating mean (SD)
	Fully disagree				Fully agree	
Early detection of A-T overburdens parents with information about an untreatable disease during the maternity period	7.3	19.7	13.4	42.4	16.4	3.4 (1.19)
Early detection of A-T deprives parents of the opportunity to enjoy a seemingly healthy baby in the first months/years of life	5.5	20.7	18.3	38.4	16.5	3.4 (1.15)
Early detection of AT makes parents worry about the disease before the symptoms even occur	9.0	27.9	15.8	38.4	8.1	3.0 (1.16)
Every child has the right to an open future	11.1	24.3	31.2	21.5	10.6	3.0 (1.16)
Early detection of A-T overburdens parents with information about the increased risk of breast cancer for the mother during the maternity period	10.6	33.5	15.6	30.0	9.4	2.9 (1.20)
Early detection of A-T adds little to the quality of life of a child with A-T	12.6	44.9	20.0	17.2	4.7	2.6 (1.06)
The disease A-T cannot be prevented or treated anyway	19.8	37.8	18.1	18.4	4.7	2.5 (1.14)
You have to take life as it comes	19.8	31.5	28.2	14.5	5.0	2.5 (1.06)
Early detection of A-T can lead to a reduced bond between parents and child	38.7	29.5	15.4	11.5	4.5	2.1 (1.18)

SD = Standard deviation. [a] Five-point rating scale: 1 = fully disagree; 5 = fully agree; N = 659 respondents. Missing values are excluded from the percentages.

Intention to participate in A-T screening and opinion on current policy for NBS for SCID
In total, 288 of the parents (44%) would participate in A-T screening if a test would be available (as they indicated 'yes' to this hypothetical question). In addition, 234 of the parents (37.1%) intended to participate in screening for A-T if a test would be available (indicated by 'probably yes'). The two main decisive arguments to participate were: 'early detection of A-T prevents a long period between the first symptoms and the diagnosis' and 'early detection of A-T ensures that a child with A-T can immediately receive optimal guidance when the first symptoms occur'. In total, 16 parents (2.4%) did not intend to participate in screening for A-T. Moreover, 47 parents (7.2%) would probably not participate in screening for A-T. The main decisive argument to decline screening for A-T was: 'early detection of A-T deprives parents of the opportunity to enjoy a seemingly healthy baby in the first months/years of life'. In the case of an abnormal screening result for SCID, 81.9% (N = 538) of the parents think that diagnostics for A-T should be applied. In addition, the majority of parents (72.9%/N = 478) disagrees with the current NBS for SCID protocol in which A-T diagnostics are not applied after abnormal SCID screening results, but only if symptoms of A-T start to occur. The opinion of parents of A-T patients, as described recently by Schoenaker et al. (2020) did not differ from our research population with regard to this policy (P = 0.403). Parents of A-T patients were less convinced that A-T should be added to the NBS program if a test was available in comparison to parents of healthy newborns (76% versus 91.4% respectively) (Table 6).

Multivariate logistic regression regarding newborn screening for A-T
The only variable with a significant association to the outcome variables was 'the number of children' (Table 7). Respondents who had their first child (number of children 1) were more likely to participate in NBS for A-T than respondents with more children (number of children >1). Parents with one child were also more likely to believe that A-T should be added to the NBS program. Other variables (age, gender, ethnicity, level of education, having a child with a (genetic) condition and having a family member with a hereditary disorder) were not significantly associated with any of the outcome variables (Table 7).

Table 6. Comparison to the perspective of parents of A-T patients: opinions on current policy and NBS for A-T

Survey question:
In the case of an abnormal SCID screening result that turns out not be SCID after follow-up diagnostics, diagnostics for A-T should not be applied. Additional diagnostics for A-T should only be used if symptoms of A-T begin to occur.
If a technique was available that would be able to detect all children with A-T with NBS, A-T should be included in the NBS program.

SD = Standard deviation. [a] Five-point rating scale: 1 = fully disagree; 5 = fully agree; Missing values are excluded from the percentages. [b] Data collected via the questionnaire send to parents of A-T patients [20].[c] χ^2 test. [d] N = 77 answered 'don't know' and were excluded from analysis.

Table 7. Multivariate logistic correlation

Predictor variable
Age (20-30 years)
Gender (female)
Ethnicity (Dutch)
Educational level (high)
Number of children (first child)
Having a sick child (yes)
Having a family member with a hereditary disease (yes)

Multivariate logistic regression analyses (N =581 valid cases) with standardized regression coefficients β and standard error (SE). Missing values were excluded from the multivariate regression analysis.

Parents of A-T patients Degree of support [a] N(%) Total N = 35 [b]					Parents of healthy newborns Degree of support [a] N(%) Total N = 659					P-value [c]
Fully disagree				Fully agree	Fully disagree				Fully agree	
12 (37.5)	12 (37.5)	3 (8.8)	6 (17.4)	1 (2.9)	150 (22.9)	328 (50.0)	61 (9.3)	90 (13.7)	27 (4.1)	0.403
Missing 1					Missing 3					
No		Yes			No		Yes			
8 (24%)		25 (76%)			49 (8.6%)		523 (91.4%)			**0.03**
Missing 2					Missing 10[d]					

A-T should be added to NBS program			Intended participation NBS A-T		
B	**SE**	**P**	**B**	**SE**	**P**
1.001	0.617	0.105	-0.205	0.608	0.736
0.409	0.488	0.402	-0.137	0.409	0.738
-1.327	0.743	0.74	1.090	0.612	0.075
-0.11	0.382	0.977	-15.927	1929.242	0.736
17.173	**0.623**	**0.0001**	**16.097**	**0.653**	**0.0001**
0.480	0.786	0.374	0.138	0.781	0.860
-15.998	5102.717	0.997	16.322	5405.408	0.998

DISCUSSION

The aim of this study was to provide insight into parents' perspectives about the early detection of A-T and with that to collect empirical data on public acceptance of untreatable findings to NBS. The vast majority of parents in our study population believed that advantages of early detection of A-T outweighed the disadvantages (81.9%). The prevention of a long period between first symptoms and diagnosis and the fact that early detection will ensure that a child with A-T can immediately receive optimal guidance when the first symptoms occur were the most important arguments from their perspective. Parents who see more disadvantages than advantage in early detection of A-T (9.6%) believe that early detection of A-T deprives parents of the opportunity to enjoy an apparent healthy baby in the first months/years of life. The public attitude towards reporting A-T as an untreatable incidental finding of NBS for SCID thus appeared to be positive. In the case of an abnormal screening result for SCID, 81.9% of the parents think that diagnostics for A-T should be applied. In addition, the majority of parents (72.9%) disagree with the current NBS for SCID protocol in which A-T diagnostics are not applied after abnormal SCID screening results, but only if symptoms of A-T start to occur.

The perspective of parents of healthy newborns is a reflection of the public, but the opinions of parents of patients are of great importance as well. Both parents of healthy newborns and parents of A-T patients favored the advantages of early detection of A-T in the asymptomatic phase over the disadvantages [20]. Decisive arguments differed amongst groups; whereas parents of healthy newborns valued the optimal clinical guidance from the start, parents of a child with A-T mentioned the uncertainty towards the diagnosis and the impact on their lives. This last argument would be difficult to envision for parents of healthy newborns, as they have not experienced it first-hand. Parents of A-T patients additionally mentioned the importance of knowledge about the inheritance and recurrent risk of A-T when making reproductive choices [20]. Both parents of healthy newborns and parents of A-T patients who were opposed to early detection of A-T valued the 'happy/golden years'. These findings suggest that first-hand experience with the untreatable disorder is an independent factor in the final opinion of parents on early detection of this disorder, although the arguments used are colored by these experiences.

The discussion about reporting untreatable incidental findings goes hand in hand with the discussion about NBS for untreatable disorders. There is a difference in actively screening for untreatable disorders and reporting them as incidental findings to NBS for treatable disorders. In this study, both aspects were studied amongst parents: the

situation of A-T as untreatable disorder to NBS for SCID discussed previously and the hypothetical situation of NBS for A-T. The result showed high support for neonatal screening for A-T in the general public. The support was consistent for both the public health perspective (should A-T be added to the neonatal screening program?) and the personal perspective (would you use the screening?). The great majority of parents would (probably) participate in NBS for A-T if a test would be available. Moreover, most parents were convinced that A-T should be added to the NBS program if a test was available. These findings are in direct contradiction to the Wilson and Jungner criteria (1968) which state the screened disorders should have an available treatment. Remarkably, results indicated that parents with (only) one child were more likely to participate in NBS for A-T than respondents having more children. This group was also more likely to believe that A-T should be added to the NBS program. These findings suggest that 'new' parents have a higher support for NBS for A-T than parents with children who are somewhat older and are likely to be more experienced in parenting. A possible explanation could be that feelings of uncertainty that are accompanied with new parenthood, makes parents look for ways of health confirmation, such as participation in additional screening programs [21].

In addition to parents of healthy newborns, the majority of parents of A-T patients were in favor of adding A-T to the NBS program as well. This implies a high level of support for NBS for A-T, not only among those who have personal experience of the disease but also among the general public. In the past, patient organizations have promoted the expansion of NBS for particular conditions, while evidence-based reviews by professional experts have been more hesitant [22]. Our findings are similar to studies about NBS for other (previously considered as) untreatable disorders. The study of Weinreich et al. (2012) compared the perspective of a consumer panel with (parents of) patients with Pompe disease. In total, 87% of the consumer panel and 88% of the Pompe group supported the introduction of NBS for Pompe [16]. The study of Wood et al. (2014) showed high support amongst parents of children with Duchenne and Becker Muscular Dystrophy and Spinal Muscular Atrophy (SMA) for NBS for these conditions. Of their survey cohort, 95.9% of believed that NBS should be implemented, even in the absence of therapeutic consequences [23]. These findings can also be extrapolated to the opinion of the general public. In the United Kingdom, a survey study revealed that 84% of participants from the general public were in favor of NBS for SMA, compared to 70% support among SMA families [24]. In the meantime, treatment for SMA became available and in July 2019, the Health Council of the Netherlands deemed SMA to be a suitable candidate to be included in the Dutch NBS program [25]. Focus groups amongst a diversity of mothers with young children showed great support for NBS for untreatable conditions presenting in infancy. Similar arguments to our study

population were mentioned such as the importance of emotional preparation and the avoidance of the 'diagnostic Odyssey' [26]. Furthermore, in the study of Hayeems et al. (2015) the majority of participations in focus groups supported NBS for serious disorders for which treatment is not available (95-98, 82%).Anticipated benefits of expanded infant screening were prioritized over harms [27]. However, the authors urged caution around the potential for public enthusiasm to foster unlimited uptake of infant screening technologies.

The perspective of parents as key stakeholders in NBS is of great value for policymaking. While some countries embrace all incidental findings, the current policy in the Netherlands on reporting untreatable incidental findings is more conservative. Cultural and moral believes seem to be of influence in the decision making process around screening and reporting of untreatable (incidental) findings. Expanding our study to other countries who have implemented NBS for SCID would create an interesting opportunity to study the influence of these believes on parents' perspective on screening for untreatable disorders. Policy makers need to balance different perspectives and needs in discussion about NBS for untreatable disorders, such as high quality evidence, benefits or harms for the routine screening program, costs, values of the population as well as contextual considerations. The Health Council of the Netherlands stated in 2015 that some benefits of screening/reporting for untreatable (incidental) disorders such as shortening the diagnostic process and the ability to adapt/prepare to a life with a condition might be in the interest of the child. In addition, a long-term diagnostic process can have negative effects on the psychological well-being of a child and his or her family [15]. However, as it is not self-evident that screening for untreatable disorders is in the best interest of the child and as empirical data on the advantages and disadvantages of early knowledge of untreatable disorders are limited, the discussion in the Netherlands is ongoing. Without scientific evidence that neonatal screening can prevent significant health damage, the Council states that extending the NBS program with untreatable diseases would be undesirable [15]. Our results as well as other studies that showed support for the screening of untreatable disorders will serve as valuable tools and scientific evidence in advising policymakers in their considerations about NBS for non-treatable disorders.

This study encountered several strengths and limitations. The questionnaire was sent to a large number of parents thereby increasing the external validity of the study. Moreover, the use of a sequential mixed methods approach and open coding by two different researchers (MB and MH) increased the internal validity and enhanced a deeper understanding of the subject. The ability to compare our study data of parents of healthy newborns with the data of parents of A-T patients [20] provides a complete

overview of the perspective of different groups of parents on the early detection of A-T in a pre-symptomatic phase. In additions to these strengths, the study has some limitation. The research population is significantly different from the Dutch reference population and may therefore not completely reflect the attitude of the general Dutch population. Some parents indicated that the questions could be experienced as too difficult which could result in bias towards higher educated respondents. In addition, the participants in the study were chosen among those who voluntary participated in the SONNET study. This could potentially create a study population biased towards favoring NBS for any disorder. As the objection rate in the SONNET-study was only 0.6%, (data not published), bias is limited and the results of this questionnaire study would still reflect the perspective of the majority of parents. Finally, the study has a relatively low response rate. Previous studies indicate that a low response rate does not automatically mean the study results have low validity [28], they simply indicate a potentially greater risk of this. This study reports methods of recruitment and provides detailed information about the respondents increasing the validity and utility of the study results. The response rate could be improved if a reminder was allowed to be sent [29].

CONCLUSION

Reporting untreatable incidental findings remains a disputed topic worldwide. The current policy in the Netherlands is to not report these incidental findings, unless early detection prevents significant health damage to the child. The majority of parents of healthy newborns are in favor of an early A-T diagnosis in the pre-symptomatic phase of the disease. Moreover, the majority of parents would use a screening test for A-T, if such a test were available. Decisive arguments to participate were the fact that early detection of A-T prevents a long period between the first symptoms and the diagnosis and that early detection of A-T ensures immediate optimal guidance for a child when the first symptoms occur. With the ongoing discussion in the Netherlands on reporting untreatable incidental findings and NBS for untreatable diseases, parent's perspective could be used as a valuable tool for policy-makers who aim to balance advantages and disadvantages of early detection of rare hereditary disorders.

DECLARATIONS

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MB, MS and MvdB designed the study; MB, MV, CW, MW and LH developed the questionnaire; MB and MH collected and analyzed the data; MB, MH and MvdB wrote the paper; all authors edited the paper and approved the final version.

Funding

This study was supported by The Netherlands Organisation for Health Research and Development ZonMW (SONNET study, project 543002002) and the Action for A-T foundation (project 4519). LH received funding from ZonMw to study the psychosocial aspects of (expanded) NBS (PANDA study, project 543002006).

Acknowledgments

The authors thank all parents for their participation in this study. Authors would like to thank the team from the RIVM-archive for digitalizing the questionnaires and a special thanks to Lisette Burger (Department of Biologicals, Innovation and Screening – RIVM) who helped a great deal with data entry.

REFERENCES

1. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2).
2. Rechavi, E., et al., First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol, 2017. 8: p. 1448.
3. Kwan, A., et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama, 2014. 312(7): p. 729-38.
4. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol, 2005. 115(2): p. 391-8.
5. Thomas, C., et al., Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clin Immunol, 2019. 202: p. 33-39.
6. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
7. Dorsey, M. and J. Puck, Newborn Screening for Severe Combined Immunodeficiency in the US: Current Status and Approach to Management. Int J Neonatal Screen, 2017. 3(2).
8. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunol Rev, 2019. 287(1): p. 241-252.
9. Rothblum-Oviatt, C., et al., Ataxia telangiectasia: a review. Orphanet journal of rare diseases, 2016. 11(1): p. 159-159.
10. van Os, N.J.H., et al., Ataxia-telangiectasia: recommendations for multidisciplinary treatment. Dev Med Child Neurol, 2017. 59(7): p. 680-689.
11. van Os, N.J., et al., Health risks for ataxia-telangiectasia mutated heterozygotes: a systematic review, meta-analysis and evidence-based guideline. Clin Genet, 2016. 90(2): p. 105-17.
12. Weigelt, B., et al., The Landscape of Somatic Genetic Alterations in Breast Cancers From ATM Germline Mutation Carriers. J Natl Cancer Inst, 2018. 110(9): p. 1030-1034.
13. Mallott, J., et al., Newborn screening for SCID identifies patients with ataxia telangiectasia. J Clin Immunol, 2013. 33(3): p. 540-9.
14. Borte, S., et al., Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood, 2012. 119(11): p. 2552-5.
15. Health Council of the Netherlands, Neonatal screening: new recommendations. 2015, Health Council of the Netherlands: The Hague.
16. Weinreich, S.S., et al., Public support for neonatal screening for Pompe disease, a broad-phenotype condition. Orphanet J Rare Dis, 2012. 7: p. 15.
17. Statistics Netherlands. Population; key figures. 2018 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37296eng/table?ts=1564487099871.
18. Statistics Netherlands. Labour Force; level of education by personal characteristics. 2019 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/71822eng/table?fromstatweb.
19. Statistics Netherlands. Households; size, composition, position in the household, 1 January. 2018 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/82905ENG/table?ts=1564487413252.
20. Schoenaker, M.H.D., et al., Early diagnosis of Ataxia Telangiectasia in the neonatal phase: a parents' perspective. Manuscript submitted for publication, 2019.

21. Wiklund, I., et al., New parents' experience of information and sense of security related to postnatal care: A systematic review. Sex Reprod Healthc, 2018. 17: p. 35-42.
22. Levy, P.A., An overview of newborn screening. J Dev Behav Pediatr, 2010. 31(7): p. 622-31.
23. Wood, M.F., et al., Parental attitudes toward newborn screening for Duchenne/Becker muscular dystrophy and spinal muscular atrophy. Muscle Nerve, 2014. 49(6): p. 822-8.
24. Boardman, F.K., C. Sadler, and P.J. Young, Newborn genetic screening for spinal muscular atrophy in the UK: The views of the general population. Mol Genet Genomic Med, 2018. 6(1): p. 99-108.
25. Health Council of the Netherlands, Neonatal screening for spinal muscular atrophy. 2019, Health Council of the Netherlands: The Hague.
26. Hasegawa, L.E., et al., Parental attitudes toward ethical and social issues surrounding the expansion of newborn screening using new technologies. Public health genomics, 2011. 14(4-5): p. 298-306.
27. Hayeems, R.Z., et al., Expectations and values about expanded newborn screening: a public engagement study. Health Expect, 2015. 18(3): p. 419-29.
28. Morton, S.M.B., et al., In the 21st Century, what is an acceptable response rate? 2012. 36(2): p. 106-108.
29. Nakash, R.A., et al., Maximising response to postal questionnaires--a systematic review of randomised trials in health research. BMC Med Res Methodol, 2006. 6: p. 5.
30. Statistics Netherlands. Birth; key figures. 2017 [cited 2019 15 July]; Available from: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37422eng/table?ts=1564486871296.

SUPPLEMENTARY MATERIAL
QUESTIONNAIRE

Early diagnosis of Ataxia Telangiectasia

This questionnaire consists of five different sections:

- Section A. Background information about Ataxia Telangiectasia (A-T)
- Section B. Scenarios of two A-T patients
- Section C.Statements about early detection of A-T
- Section D. Final questions
- Section E.Demographics

A. Background information

Ataxia Telangiectasia (A-T) is a rare, serious disease. This disease causes serious, progressive neurological symptoms, such as problems with balance and coordination. Patients with A-T have more frequent infections and an increased risk of developing cancer, in particular of the blood and lymph nodes (leukemia or lymphomas). Children with A-T have a shorter life expectancy. In addition, mothers of A-T patients have a slightly increased risk of developing breast cancer. A-T is a hereditary disease.

This means that other children within the same family could also have A-T.

There is no cure for A-T and it is not possible to delay the disease-onset or progression. The treatment is aimed at treating the various symptoms of the disease. For example, children with A-T often receive physiotherapy and come to the hospital for a check-up with the pediatrician once a year. As A-T is a rare disease and not all characterizing symptoms might be present in each case, it sometimes takes doctors quite some time to think about the diagnosis A-T.

B. Scenarios and questions

If a newborn has an abnormal newborn screening result for SCID, the newborn will be referred for additional confirmatory diagnostics in the (academic) hospital. An abnormal screening result means that the child might have SCID. The child could also have another disease with an immune disorder such as A-T. Children with A-T are asymptomatic at birth. In this section, two stories of children with A-T are described, followed by questions.

- Early diagnosis of A-T or early detection of A-T means that the diagnosis A-T is directly made after birth. The child has no symptoms at that time.
- Late diagnosis of A-T or late detection of A-T means that the diagnosis A-T is made later in life when the child has already developed symptoms.

Scenario 1. Max and a late diagnosis of A-T

Max is three days old when newborn screening is performed. The newborn screening result for SCID is abnormal. A pediatrician in the hospital performs additional medical examinations. Max does not have SCID. No additional diagnostics is done for Ataxia Telangiectasia and Max is allowed to go home. When Max is 12 months old, he has difficulty with crawling. The youth healthcare doctor thinks that Max is just developing a little bit slower compared to his age group. When Max is 20 months old, he starts walking, but falls over a lot. He also often has colds. Max's mother is worried. The GP thinks that Max's walking will improve in the future. When Max continues to fall over and his walking does not improve, Max's parents are referred to the hospital. Max is 4 years old by that time and now has a little brother of 6 months old. The pediatrician performs additional diagnostics and diagnoses Max with Ataxia Telangiectasia (A-T). Max's parents are told that A-T cannot be cured. Max can end up in a wheelchair and there is a chance that he will develop cancer at a young age. His life expectancy is shorter compared to other children. Max's mother is referred for early breast cancer screening. Additional diagnostics are being done to test if Max's little brother also has A-T.

1. What do you believe to be the advantages of late detection of A-T in Max's case?

2. What do you believe to be the disadvantages of late detection of A-T in Max's case?

Scenario 2. Lotte and an early diagnosis of A-T

Lotte is three days old when newborn screening is performed. The newborn screening result for SCID is abnormal. A pediatrician in the hospital performs additional medical examinations. Lotte does not have SCID. Additional diagnostics are done for Ataxia Telangiectasia. After three weeks, Lotte's parents receive the results of the additional tests: Lotte has Ataxia Telangiectasia. Lotte is a seemingly healthy baby girl without symptoms at the time of diagnosis. A pediatrician explains to Lotte's parents that A-T cannot be cured. Lotte can end up in a wheelchair and has a shorter life expectancy compared to other children. Lotte's mother finds it difficult to process all this information. She is still recovering from child-birth. Since Lotte currently has no symptoms, physical therapy will not yet be started. However, from now on Lotte will be closely monitored by an experienced team of medical specialists. This team of specialist will immediately check-up on Lotte when the first symptoms of A-T occur. Lotte's mother is referred for early breast cancer screening.

3. What do you believe to be the advantages of early detection of A-T in Lotte's case?

4. What do you believe to be the disadvantages of early detection of A-T in Lotte's case?

C. Statements about early detection of A-T

- Early diagnosis of A-T or early detection of A-T means that the diagnosis A-T is directly made after birth. The child has no symptoms at that time.
- Late diagnosis of A-T or late detection of A-T means that the diagnosis A-T is made later in life when the child has already developed symptoms.

1. A number of advantages of early detection of A-T are stated below. Please indicate which box reflects your opinion best.

A-T <u>should be</u> detected early, because:

	Fully disagree	Disagree	Neither agree, nor disagree	Agree	Fully agree
Early detection of A-T prevents a long period betweenthe first symptoms and the eventual diagnosis	☐	☐	☐	☐	☐
Early detection of A-T prevents multiple visits tothe hospital	☐	☐	☐	☐	☐
Early detection of A-T saves extra health costs	☐	☐	☐	☐	☐
Early detection of A-T prevents a long period ofuncertainty for parents	☐	☐	☐	☐	☐
Early detection of A-T prevents unnecessaryadditional tests	☐	☐	☐	☐	☐
Early detection of A-T provides parents with the opportunity to make informed choices about familyplanning	☐	☐	☐	☐	☐
Early detection of A-T ensures that a child with A-Tcan immediately receive optimal guidance when the first symptoms occur	☐	☐	☐	☐	☐
It is an advantage to be informed about the slightly increased risk of developing breast cancer for mother	☐	☐	☐	☐	☐
Early detection of A-T ensures that parents can adjusttheir expectations about the condition of their child	☐	☐	☐	☐	☐
Early detection enables parents to make earlyadjustments into their lives (e.g. wheelchair accessible house)	☐	☐	☐	☐	☐
Early detection of A-T ensures that parent can take better care of their child	☐	☐	☐	☐	☐

2. A number of disadvantages of early detection of A-T are stated below. Please indicate which box reflects your opinion best.

A-T <u>should not</u> be detected early, because:

	Fully disagree	Disagree	Neither agree, nor disagree	Agree	Fully agree
Early detection of A-T adds little to the qualityof life of a child with A-T	☐	☐	☐	☐	☐
Early detection of A-T overburdens parents with information about an untreatable disease during the maternity period	☐	☐	☐	☐	☐
Early detection of A-T deprives parents of the opportunity to enjoy a seemingly healthy babyin the first months/years of life	☐	☐	☐	☐	☐
You have to take life as it comes	☐	☐	☐	☐	☐
Early detection of A-T overburdens parents with information about the increased risk of breast cancer for mother during the maternity period	☐	☐	☐	☐	☐
The disease A-T cannot be prevented or treated anyway	☐	☐	☐	☐	☐
Early detection of AT makes parents worry about the disease before the symptoms even occurred	☐	☐	☐	☐	☐
Early detection of A-T can lead to a reducedbond between parents and child	☐	☐	☐	☐	☐
Every child has the right to an open future	☐	☐	☐	☐	☐

4. Two statements are presented below. Please indicate which box reflects your opinion best.

Statement 1. In the case of an abnormal SCID screening result, diagnostics for A-T should be applied immediately

Fully disagree	Disagree	Neither agree, nordisagree	Agree	Fully agree
☐	☐	☐	☐	☐

Statement 2. In the case of an abnormal SCID screening result that turns out not be SCID after follow-up diagnostics, diagnostics for A-T should not be applied. Additional diagnostics for A-T should only be used if symptoms of A-T begin to occur

Fully disagree	Disagree	Neither agree, nordisagree	Agree	Fully agree
☐	☐	☐	☐	☐

D. Final questions

1. It is not yet possible to detect all patients with A-T with newborn screening. However, if a test would be available to screen all newborns for A-T, would you personally participate in this screening?

No	Probably not	Don't know	Probably yes	Yes
☐	☐	☐	☐	☐
Continue to question 2	Continue to question 2		Continue to question 3	Continue to question 3

If you have answered 'don't know', you can skip questions 2 and 3 and continue to question 4.

2. What would be the decisive argument for you to not participate in newborn screening for A-T?

- ☐ Early detection of A-T adds little to the quality of life of a child with A-T
- ☐ Early detection of A-T overburdens parents with information about an untreatable disease during the maternity period
- ☐ Early detection of A-T deprives parents of the opportunity to enjoy a seemingly healthy baby in the first months/years of life
- ☐ You have to take life as it comes
- ☐ Early detection of A-T overburdens parents with information about the increased risk of breast cancer for the mother during the maternity period
- ☐ The disease A-T cannot be prevented or treated anyway
- ☐ Early detection of AT makes parents worry about the disease before the symptoms even occur
- ☐ Early detection of A-T can lead to a reduced bond between parents and child
- ☐ Every child has the right to an open future
- ☐ Other, please specify:

3. What would be the decisive argument for you to participate in newborn screening for A-T?

- ☐ Early detection of A-T prevents a long period between the first symptoms and the eventual diagnosis
- ☐ Early detection of A-T prevents multiple visits to the hospital
- ☐ Early detection of A-T saves extra health costs
- ☐ Early detection of A-T prevents a long period of uncertainty for parents
- ☐ Early detection of A-T prevents unnecessary additional tests
- ☐ Early detection of A-T provides parents with the opportunity to make informed choices about family planning
- ☐ Early detection of A-T ensures that a child with A-T can immediately receive optimal guidance when the first symptoms occur
- ☐ It is an advantage that parents are informed about the slightly increased risk of developing breast cancer for the mother
- ☐ Early detection of A-T ensures that parents can adjust their expectations about the condition of their child
- ☐ Early detection enables parents to make early adjustments into their lives (for example wheelchair accessible house)
- ☐ Early detection of A-T ensures that parent can take better care of their child
- ☐ Other, please specify:

4. If a technique was available that would be able to detect all children with A-T with newborn screening, do you think A-T should be included in the newborn screening program?

☐ Yes

☐ No

☐ Don't know

E. Demographics

1. I am…
   ☐ Male

   ☐ Female

2. What is your age?

3. What is your *highest* level of education?
   ☐ None, primary school

   ☐ LBO, MAVO

   ☐ VMBO

   ☐ MBO, HAVO, VWO

   ☐ HBO, University

   ☐ Other:

4. What is your marital status?
   - ☐ Single
   - ☐ Living together/married
   - ☐ Other:

5. In which country were you born?

6. In which country was your father born?

7. In which country was your mother born?

8. How many children do you have?

Please answer for each child:

	How old is your child today?	Was newborn screening performed in the Netherlands?	What were the results of the newborn screening program? Options: good/not good/I'd rather not say
1st child			
2nd child			
3rd child			
4th child			
5th child			

9. Are you children healthy? If no, please specify why not.
   - ☐ Yes
   - ☐ No:
   - ☐ I'd rather not say

10. Do you have a family member with a hereditary disorder? If yes, please specify the disorder in question.
   - ☐ No
   - ☐ Yes:
   - ☐ I don't know
   - ☐ I'd rather not say

11. If you have any additional comments about the questionnaire, please leave them below:

Thank you for your cooperation!

CHAPTER 9

Economic evaluation of different screening strategies for SCID based on real-life data

M. Elske van den Akker-van Marle, Maartje Blom, Mirjam van der Burg, Robbert G.M. Bredius and C.P.B. (Kitty) van der Ploeg

International Journal of Neonatal Screening, 2021; 7(3): 60

ABSTRACT

Although several countries have adopted severe combined immunodeficiency (SCID) into their NBS program, other countries are still in a decision process of adding this disorder in their program and finding the appropriate screening strategy. This decision may be influenced by the cost(-effectiveness) of these screening strategies. In this study the cost(-effectiveness) of different newborn screening (NBS) strategies for SCID were estimated based on real-life data from a prospective implementation study in the Netherlands. The cost of testing per child for SCID was estimated at €6.36. Cost of diagnostics after screen-positive results were assessed to vary between €985 and €22 8561 per child dependent on final diagnosis. Cost-effectiveness ratios varied from €41,300 per QALY for the screening strategy with T-cell receptor excision circle (TREC) ≤ 6 copies/punch to €44,100 for the screening strategy with a cut-off value of TREC ≤ 10 copies/punch. Analysis based on real-life data results in higher costs, and consequently in less favorable cost-effectiveness estimates than analyses based on hypothetical data, indicating the need for verifying model assumptions with real-life data. Comparison of different screening strategies suggest that strategies with a lower number of referrals, e.g., by distinguishing between urgent and less urgent referrals, are favorable from an economic perspective.

INTRODUCTION

Newborn screening (NBS) aims at detecting conditions shortly after birth that are treatable, but not clinical evident in the newborn period. By detecting these conditions in an early phase, clinical manifestation of the disease may be prevented, or the course of the disease might be influenced positively. NBS was first introduced in the United States in the early 1960s using screening cards with dried blood spots [1], and has expanded to countries around the world, while also the number of conditions included in NBS programs is growing.

One of the most life-threatening inherited disorders of the immune system is severe combined immunodeficiency (SCID). Patients with SCID are usually born asymptomatic, but present with severe, recurrent infections, chronic diarrhea and failure to thrive in the first months of life. Without curative treatment in the form of hematopoietic stem cell transplantation (HSCT) or gene therapy, fatal outcome is inevitable [2]. Previous studies showed that early detection and treatment of SCID patients in the pre-symptomatic phase is associated with improved outcomes and higher survival rates [3-5]. Particularly an infection-free status at the time of HSCT is important, herewith highlighting the importance of early detection and protective management to prevent infections.

Early detection of SCID can be realized via NBS by the detection of T-cell receptor excision circles (TRECs) in dried blood spots with quantitative polymerase chain reaction (qPCR) [6, 7]. TRECs are circular DNA fragments formed during the T-cell receptor gene rearrangement. Absence of TRECs is indicative for the absence of recently formed naïve T-cells. There is a range of neonatal conditions and disorders that can be associated with T-cell lymphopenia and low TRECs around birth that are not related to SCID. Low or absent TRECs can also be identified in preterm newborns, newborns with congenital malformation or T-cell impairment syndromes [8, 9]. These findings can be considered secondary findings of NBS for SCID. To distinguish SCID from other T-cell lymphopenias, follow-up diagnostics by flow cytometric immunophenotyping and genetic analysis are indicated.

Although several countries have adopted SCID into their NBS program, one of the issues that remains to be solved is finding the appropriate screening strategy that balances a high sensitivity and avoiding missing neonates with SCID, while preventing high referral rates and a high number of secondary findings. A high referral rate is associated with a high emotional impact for parents, high workloads for downstream referral centers and high diagnostic costs. Therefore, decisions have to be made on the appropriate TREC cut-off value and screening algorithm.

Whereas some countries are optimizing their screening strategy for NBS for SCID, other countries are still in an ongoing discussion about implementation of this disorder in their program. The decision of adding a disease to the NBS program may be influenced by a cost-effectiveness analysis in which the additional effects of screening are related to the costs compared to a situation without screening. In cost-effectiveness studies on adding SCID to the NBS program from the USA, New Zealand and UK, cost-effectiveness ratios (CER) ranged from €19,000 to €44,000 per quality of life-year (QALY) gained [10-14]. A Dutch study for a hypothetical cohort based on literature estimates and expert opinion resulted in a CER of €33,400 per QALY gained, suggesting that SCID screening in the Netherlands might be cost-effective but pilot studies are warranted to reduce uncertainty around the estimates [15]. In the Netherlands a prospective implementation pilot study on NBS for SCID (SONNET-study) started in April 2018, with the aim to gather knowledge about the practical implications of NBS for SCID, test qualities, costs and the perspective of users (i.e., health care providers and parents). In this study, the costs of screening and diagnostics for different NBS strategies for SCID were assessed based on real-life data from the prospective implementation study. Furthermore, the previously used model was updated with these data to explore the consequences for the estimates of the iCER of SCID screening compared to a situation without screening.

METHODS

Prospective implementation pilot

For the SONNET-study, all parents of newborns born in three of the twelve provinces of the Netherlands (Utrecht, Gelderland and Zuid-Holland) were asked to participate in a research project on NBS for SCID (opt-out consent). All dried blood spots (DBS) included were collected as part of the Dutch routine NBS program from April 2018 onwards. The SONNET-study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

TREC analysis

TREC analysis was performed according to the SPOT-it™ kit instructions for use (ImmunoIVD, Stockholm, Sweden) according to a preset screening algorithm ([16]. NBS cards with TRECs below cut-off required repeated analysis in duplicate (retest). Full term infants with repeated TREC levels below cut-off had an abnormal screening result and were referred for follow-up diagnostics. Preterm infants with abnormal results required a second specimen to be collected from the corrected gestational age of 37 weeks (second heel prick). Abnormal screening results with low β-actin levels were considered inconclusive and required repeated sampling (repeated first heel prick).

Adjusted cut-off values and new screening algorithm (post hoc)
From April 2018 to October 2018, newborns with TREC ≤ 6 copies/3.2 mm punch were referred for clinical follow-up, according to the kit instructions of the manufacturer (ImmunoIVD). After six months of screening, the TREC cut-off value was increased to ≤ 10 copies/3.2 mm punch to ensure that no atypical SCID cases would be missed. For this study, the adjustment in cut-off value allows the investigation of both screening situations (cut-off TREC ≤ 6 and ≤ 10) as all newborns from November 2018 with TRECs ≤ 10 were referred for follow-up diagnostics. Screening data was included from 1 November 2018 to 31 July 2020 (N = 127,160 screened newborns).

There are a number of medical conditions without an intrinsic defect in the number of T-cells leading to low TREC levels around birth. Some of these conditions could resolve within the first few days to weeks after birth, leading to normalization of TRECs levels. For this reason, a new screening algorithm was developed post hoc that distinguishes between urgent referrals with TREC levels ≤ 2 copies/3.2 punch, and cases with TREC levels > 2 to ≤ 10 that require a second heel prick after seven days. Based on retrospective data of the SONNET-study, it was determined which newborns would be directly referred and which newborns would have required a second heel prick [17].

Cost of screening
Screening data from the SONNET-study were obtained via the NEONAT database, the national laboratory information system in which all NBS test results are stored. The numbers of first heel pricks, duplicate analyses and second duplicate analyses (all on the first blood sample) were obtained, as well as the numbers of repeated first heel pricks and second heel pricks needed and performed (and duplicate analyses in these). For the new post hoc screening algorithm, the number of additional second heel pricks was determined based on the number of children with TREC > 2 to ≤ 10. In the cost calculations, we assumed all would have been performed.

Costs of screening test were assessed using the microcosting approach, by collecting detailed data on resources utilized and the value of those resources [18]. The price level of 2020 was used (€2020). Cost of screening consists of costs of the TREC assay, use of laboratory equipment, material and personnel costs. Cost of the assay were based on the arrangement between the manufacturer and Dutch screening laboratories. These included lease of the thermal cycler and qPCR instrument. Costs of other equipment was obtained by straight-line depreciation of the equipment needed in each of the five screening laboratories in the Netherlands, assuming a lifetime of 5 years, maintenance costs and interest and a nationwide use of 170,000 times a year [19]. Also, yearly cost of additional laboratory personnel (laboratory technician 0.6 fte/lab, scientific staff 0.1

fte/lab, 5 labs) needed for SCID screening was divided by the yearly number of SCID tests, to obtain personnel costs per test. The cost of blood collection and logistics were not included for the first test, as heel prick blood samples are already processed for other screening purposes. In case a repeated first heel prick or a second heel prick sample is needed for SCID, cost of blood collection and logistics were included.

In some cases, test results indicated the need for an additional heel prick, but this was not performed, e.g., because the child passed away before the heel prick could be performed. Costs were only accounted for when the heel prick was actually performed. For the new screening strategy, we assumed that all second heel pricks would have been performed.

Cost of diagnostics

Information on the diagnostic process of infants with abnormal SCID screening results were obtained from the academic hospitals participating in the SONNET study. Numbers and types of tests and clinical contacts, outpatient visits and hospital days were retrieved from the medical records of the children referred until November 2020. At that time, diagnostics were completed for the majority of the children. If not, the diagnostics that were expected to take place have been included in the analysis. Subsequently, health care use was multiplied with cost prices. Cost prices were obtained from the Dutch costing manual [19, 20] and Dutch Healthcare Authority [21].

To assess cost of diagnostics in a situation without screening, a pediatrician (RB) and clinical researcher (MB) reviewed the medical records of the infants referred and estimated which diagnostics likely would have happened in a situation without screening. Costs are reported in 2020 euros.

Cost-effectiveness

The new estimates of costs of screening and diagnostics (in a situation with and without screening) and the number children referred for the different screening strategies were included in the decision analysis model of Van der Ploeg et al. [15], to explore the con-sequences for the iCER of NBS for SCID compared to a situation without NBS for SCID. The decision analysis model used a lifetime horizon and employed the healthcare perspective. Model parameters are shown in the Supplementary Material. A more detailed description of the model and sensitivity analyses is given elsewhere [15].

RESULTS

Costs of testing on first heel pricks were determined at €6.36 per heel prick card. Costs of repeat first heel pricks and second heel pricks were estimated at € 79.03 (Table 1). Costs to refer a child are €145 (1 hour of work for a medical advisor at an hourly rate of €145).

Table 1. Cost of first, repeated first and second heel prick for severe combined immunodeficiency (SCID) in 2020€

Cost item	First heel prick	Repeated first heel prick/ Second heel prick
Blood collection[1]	[2]	€ 22.05
Postage cost	[2]	€ 0.92
Sample processing	[2]	€ 2.70
Administration	[2]	€ 47.00[3]
Testing[1]	€4.94	€ 4.94
Other equipment	€ 0.11	€ 0.11
Laboratory personnel	€ 1.28	€ 1.28
Materials	€ 0.03	€ 0.03
Total costs	€ 6.36	€ 79.03

[1] including 21% value added tax (VAT), for the T-cell receptor excision circle (TREC)-assay as well as use of the thermal cycler and quantitative polymerase chain reaction (qPCR) instrument, and including costs for retest of the same sample of newborn screening (NBS) card
[2] no additional costs compared to existing heel prick screening
[3] 0.5 hours of work at an hourly rate of €94

In the period from November 1st, 2018 to July 31st, 2020 127,160 newborns were screened for SCID. Percentages of repeat first heel pricks and second heel pricks ranged between 0.003% and 0.006%, and 0.016% and 0.061%, respectively, for the different screening strategies. Cost of screening per newborn are comparable for the different screening strategies (Table 2).

Fifty-six newborns obtained a positive screening result in the SONNET study. None of them had a family history of SCID or was diagnosed in utero. Referral rates of the different screening strategies varied between 0.022% and 0.041%. Most of the referred newborns had secondary T-cell impairment.

Table 2. Number of 1st heel pricks, repeated 1st heel pricks, 2nd heel pricks, and referrals and cost of screening based on SONNET trial in the period November 1st, 2018 to July 31st, 2020 (in 2020€)

Screening strategy	**TREC ≤ 6 copies/3.2 mm punch**	
	# (% of FHP)	€
First heel pricks (FHP)	127,160	808,367
Repeated first heel pricks	4 (0.003%)	316
Second heel pricks	20 (0.016%)	1581
Referrals	33 (0.026%)	4785
- SCID	1	
- Secondary T-cell impairment	18	
- Idiopathic lymphocytopenia	3	
- T-cell impairment syndromes	7	
- False-positive	4	
Total costs		815,048
Cost per newborn screened		6.41

[1] Direct referral if TREC levels ≤ 2 copies/3.2 punch, and cases with TREC levels > 2 to ≤ 10 require a second heel prick after seven days.

[2] For the new post hoc screening algorithm, the number of additional second heel pricks was determined based on the number of children with TREC > 2 to ≤ 10 (N = 40). In the cost calculations, we assumed all would have been performed.

Diagnostics after positive screening test consisted of personnel time during clinical contacts, during initial hospital stay, outpatient visits, consultations, emergency care visits, additional hospital stays, and diagnostic tests such as flow cytometry and whole exome sequencing using a whole exome sequencing SCID gene panel (WES SCID). Average cost per screen positive for the diagnostic procedures depend on the final diagnosis and ranged from € 985 to € 8561 (Table 3).

In a situation without screening, costs of diagnosis of SCID are assumed to be the same as in a situation with screening (€ 7517). Cost of secondary T cell impairment, idiopathic lymphocytopenia, and T-cell impairment syndromes were assessed to be lower, €486, €2250 and € 5111, respectively. Logically, there are no costs for diagnostics after false-positive screen results in a situation without screening.

TREC ≤ 10 copies/3.2 mm punch		New screening algorithm[1]	
# (% of FHP)	€	# (% of FHP)	€
127,160	808,367	127,160	808,367
8 (0.006%)	632	8 (0.006%)	632
35 (0.028%)	2766	77[2] (0.061%)	6085
52 (0.041%)	7540	28[3] (0.022%)	4060
1		1	
29		14	
6		4	
9		5	
7		4	
	819,305		819,144
	6.44		6.44

[3] Number of referrals extrapolated. Data from the SONNET-study showed that 12 out of 52 referrals (23%) would have been directly referred for follow-up diagnostics (TREC 0-2), while 40 out of 52 referrals (77%) would have required a second NBS card (TREC > 2 to ≤ 10) with the new screening algorithm. Of these 40 referrals, peripheral blood cards spotted at the time of flow cytometry (approximately one week after first DBS) were available for 26 referred newborns. These were used as if it were the outcomes of a second heel prick. For the missing 14 blood samples, outcomes of the 26 available cards were extrapolated per diagnosis or diagnostic category.

Including the observed cost for screening and diagnostics in the SCID model of Van der Ploeg et al. [15] (see Supplementary Material for comparison of new and old parameter estimates) results in comparable iCERs of €41,300 per QALY for the screening strategy with TREC ≤ 6 copies/3.2 mm punch and €41,600 per QALY for the new screening strategy. The screening strategy with TREC ≤ 10 copies/3.2 mm punch has a less favorable iCER of €44,100 per QALY (see Table 4).

Table 3. Average costs per child of diagnostic procedures and clinical care in screen positive newborns in SONNET study in the period November 1st, 2018 to July 31st, 2020 (in 2020€)

	SCID (N = 1)	**Secondary T-cell impairment** (N = 33)
Diagnostic procedures		
- Flow cytometry	472	719
- WES SCID	5459	496
- Other diagnostics	591	131
Total diagnostic procedures	***6521***	***1346***
Clinical care		
- Clinical contacts	0	15
- Outpatient visits	775	94
- Phone consults	221	40
- Emergency care	0	0
- Consultations	0	9
- Additional hospital stay	0	43
Total clinical care	***996***	***201***
Total	**7517**	**1547**
[min, max]	[7517, 7517]	[0, 7756][1]

[1] Some infants died shortly after referral, before diagnostics started.

Table 4. Model-based yearly cost and effects per 100,000 infants in a situation with and without newborn screening for SCID, and incremental cost-effectiveness ratios for different screening strategies (in 2020€)

	TREC ≤ 6 copies/3.2 mm punch
Costs of screening and additional diagnostics	671,600
- Screening	641,000
- Additional diagnostics[1]	30,600
Cost of SCID treatment	269,000
Total healthcare costs	940,600
Number of children with SCID detected	
- Early	1.72
- Late	0
QALYs gained	11.7
Cost per QALY gained	41,300

Idiopathic lymphocytopenia (N = 6)	T-cell impairment syndromes (N = 9)	False positives (N = 7)
1542	1277	612
5459	1213	0
821	914	112
7822	***3405***	***724***
0	7	0
535	320	158
332	197	103
47	95	0
21	0	0
0	2580	0
935	***3198***	***261***
8757 [6603, 12654]	**6603** [253, 23628]	**985** [655, 2024]

TREC ≤ 10 copies/3.2 mm punch	New screening algorithm	No screening
703,500	674,100	-
644,300	644,100	-
59,200	30,000	-
269,000	269,000	456,400
972,500	943,100	456,400
1.72	1.72	0.38
0	0	1.34
11.7	11.7	0
44,100	41,600	

DISCUSSION

In this study the real-life costs of testing for SCID and diagnostics after a positive screening test are used for comparing the costs of different screening strategies for SCID, and performing a model-based exploration of the cost-effectiveness of the different screening strategies.

The cost of testing per child for SCID on heel prick blood was estimated at €6.36, mainly consisting of cost of the assay and some additional costs for equipment, personnel and material. These costs are at the upper side of the range from €3.50 - €6.79 of the cost of testing reported in literature [10-12,14,15,22]. Only the upper value of €6.79 reported by Clement *et al*. [22] when assuming dedicated equipment use, i.e., using equipment exclusively for TREC analyses, was higher. The study by Clement was also based on a micro-costing study with real-life pilot data comparable to our study, which may be more reliable than hypothesized costs in other studies. Using other assays, e.g., in house methods, may lead to lower costs, but Dutch screening laboratories have strict criteria for accreditation and therefore a CE-IVD marked assay is preferred. Furthermore, the high cost of screening are also due to the fact that SCID screening is the first PCR-based test in neonatal screening programs. Implementation of this relatively new assay is associated with cost for extra equipment, reagents and extra personnel. When in a later phase other conditions such as spinal muscular atrophy (SMA) will be added to the NBS program, the TREC assay can be extended with additional primers/probes in a multiplex setting. This implies that with limited extra reagent costs screening for additional condition(s) will become possible. This will be relatively favorable for the (incremental) cost-effectiveness of these programs.

Referral rates between the screening strategies evaluated in this study varied between 0.022% and 0.041%. This is comparable to other modelling [10-14] except for our previous study in which a referral rate of 0.08% was used [15], based on referral rates found in a systematic review on TREC based screening for SCID [23].

Costs of diagnostics after screen positive results based on real-life data were assessed to vary between €985 for children with a false-positive test result to €8561 for children finally diagnosed with idiopathic lymphocytopenia. Flow cytometry and whole exome sequencing with a SCID filter (WES SCID) were major cost drivers. However, also in a situation without screening part of these costs will occur.

In addition, the costs of diagnostics of screen positive children assumed in other studies are quite low compared to our real-life estimates. Some studies only assume the need of a single appointment with diagnostic test (flow cytometry) of €209 [11,12] for screen-positive infants. McGhee *et a*l. [13] also added T cell proliferation assays, which resulted in an amount of €385. Next to assuming these presumptive positive costs consisting of an appointment and flow cytometry of €276, Bessey *et al.* [10] distinguished in cost between diagnosis: cost of SCID diagnosis were assumed to be €819 (appointment and genetic testing) and cost of diagnosis of idiopathic SCID and syndromes of €1786 (appointment and genetic testing exome panel). Van der Ploeg et al. [15] assumed the cost of diagnostics to be €1598, consisting of an appointment, flow cytometry, visit pediatrician, repeat flow cytometry for 2/3 of screen positives and genetic test for 1/3. Apparently the diagnostic procedure in practice consists of more testing and clinical care than was theoretically thought. This may be due to the fact hypothetical costs are not realistic, e.g. flow cytometry and genetic testing ask for at least two appointments, one for explaining the test and obtaining the blood sample and one for discussing the test result (which may be done by phone) while in most studies only one appointment is mentioned. Also, in practice more testing and clinical care might be performed than included in the protocols for diagnostics after a positive screening test. Last, as the whole procedure was new for care providers during this pilot study, extra diagnostic tests might have been requested to ascertain no diagnoses were missed.

However, also in a situation without NBS, diagnostic costs will be made for part of the screen positive infants. Comparing estimates for these costs for this study population to the observed diagnostic costs, additional costs of diagnostics due to screening were estimated to be €1061, €6409 and €1492 for children with secondary T cell impairment, idiopathic lymphocytopenia, and T-cell impairment syndromes, respectively. Furthermore, in some of these earlier non-SCID diagnoses a longer diagnostic trajectory may have been avoided and earlier treatment was enhanced, which may have leaded to cost savings and additional health benefits not included in this analysis. Also, our assumption that diagnostic costs for SCID are comparable in a situation with and without NBS might be conservative, as in a situation without NBS more testing might be needed to discover that the symptoms are caused by SCID.

Our real-life study leads to higher iCERs mainly due to the higher screening costs, varying from €41,300 per QALY for the screening strategy with TREC ≤ 6 copies/3.2 mm punch and €41,600 per QALY for the new screening strategy, to €44,100 for the screening strategy with cut off of TREC ≤ 10 copies/3.2 mm, compared to our earlier estimate of €33,400 per QALY based on literature data and expert opinions [14], and to

most of the other cost-effectiveness studies based on existing data, literature estimates and expert opinions, which iCERs are mainly in the range of €19,000-€29,000 per (quality-adjusted) life year gained [10-12,14]. Only one study from 2005 reported an iCER of €44,000 per QALY gained [13].

These higher iCERs obtained with real-life data are still in the range of the willingness to pay (WTP) values of 20,000 to 80,000 euros per QALY that are considered acceptable in the Netherlands [24].

From an economic point of view a screening strategy with cutoff of TREC ≤ 10 copies/3.2 mm is not preferred, while outcomes for a screening strategy with cutoff of TREC ≤ 6 copies/3.2 mm punch and the new screening algorithm are comparable. However, the new screening algorithm distinguishing in urgent referrals for TREC levels 0-2 copies/ punch and repeat heel pricks for cases with TREC levels > 2 to ≤ 10, resulted in the lowest number of referrals thereby preventing emotional stress for parents [16] and workloads for downstream referral centers, which may be arguments to prefer this screening algorithm. It is worth considering second tier test options that can reduce the number of referrals even more, although a second tier test does come with extra costs [17].

This study also has some limitations. Due to the small scale nature of pilot studies and the low referral rates, numbers of children with screen positive results are relatively low in this study, resulting in uncertainty around our estimates of diagnostic costs. As real- life estimates appeared to differ clearly from hypothetical estimates, further research is needed based on larger cohorts. Furthermore, the costs and effects of the new screening algorithm were partly based on assumptions. These assumptions have to be confirmed using real-life data. This will be possible in the future, as this screening algorithm is used in the Netherlands from January 2021 onwards. Finally, in the explorations on cost-effectiveness assumptions about the situation without screening and (long term) treatment costs and effects were still based on literature data and expert opinions. Future research is needed in which real-life estimates for these items are obtained, as this may also influence the estimates on cost-effectiveness.

In conclusion, our analysis based on real-life data results in higher costs of screening and diagnostics, and consequently in less favorable cost-effectiveness estimates for NBS for SCID than previously published analyses based on hypothetical data, indicating the need for verifying model assumptions with real-life data. Comparison of different screening strategies suggest that strategies with a lower number of referrals, e.g., by distinguishing between urgent and less urgent referrals, are favorable from an economic perspective.

Author Contributions: Conceptualization, EvdA and KvdP; methodology, EvdA and KvdP; formal analysis, EvdA and KvdP; investigation, EvdA, MB, RB, KvdP; writing—original draft preparation, EvdA; writing—review and editing, MB, MvdB, RB, KvdP; funding acquisition, MvdB, RB. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Netherlands Organization for Health Research and Development ZonMW (SONNET-study, project 543002002).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (protocol code MEC-2017-1146, 14 December 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy

Acknowledgments: The authors would like to thank all those involved in the SONNET-study for their contribution and support. The authors also thank the employees of the National Institute for Public Health and the Environment (RIVM) who provided us with information on the costs of screening.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

REFERENCES

1. Alexander D. The National Institute of Child Health and Human Development and phenylketonuria. Pediatrics. 2003;112:1514–1515.
2. Fischer A., Notarangelo L.D., Neven B., Cavazzana M., Puck J.M. Severe combined immunodeficiencies and related disorders. Nat. Rev. Dis. Primers. 2015;1:15061.
3. Heimall J., Logan B.R., Cowan M.J., et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: A PIDTC natural history study. Blood. 2017;130:2718–2727.
4. Pai S.Y., Logan B.R., Griffith L.M., et al. Transplantation outcomes for severe combined immunodeficiency, 2000–2009. N. Engl. J. Med. 2014;371:434–446.
5. Brown L., Xu-Bayford J., Allwood Z., et al. Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: The case for newborn screening. Blood. 2011;117:3243–3246.
6. Bausch-Jurken M.T., Verbsky J.W., Routes J.M. Newborn Screening for Severe Combined Immunodeficiency-A History of the TREC Assay. Int. J. Neonatal Screen. 2017;3:14.
7. Chan K., Puck J.M. Development of population-based newborn screening for severe combined immunodeficiency. J. Allergy Clin. Immunol. 2005;115:391–398.
8. Kwan A., Abraham R.S., Currier R., Brower A., et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA. 2014;312:729–738.
9. Amatuni G.S., Currier R.J., Church J.A., et al. Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010–2017. Pediatrics. 2019;143:e20182300.
10. Bessey A., Chilcott J., Leaviss J., de la Cruz C., Wong R. A Cost-Effectiveness Analysis of Newborn Screening for Severe Combined Immunodeficiency in the UK. Int. J. Neonatal Screen. 2019;5:28.
11. Ding Y., Thompson J.D., Kobrynski L., Ojodu J., Zarbalian G., Grosse S.D. Cost-Effectiveness/Cost-Benefit Analysis of Newborn Screening for Severe Combined Immune Deficiency in Washington State. J. Pediatr. 2016;172:127–135.
12. Chan K., Davis J., Pai S.Y., Bonilla F.A., Puck J.M., Apkon M. A Markov model to analyze cost-effectiveness of screening for severe combined immunodeficiency (SCID) Mol. Genet. Metab. 2011;104:383–389.
13. McGhee S.A., Stiehm E.R., McCabe E.R. Potential costs and benefits of newborn screening for severe combined immunodeficiency. J. Pediatr. 2005;147:603–608.
14. Health Partners Consulting Group Cost-Effectiveness of Newborn Screening for Severe Combined Immune Deficiency. A Report Prepared for the National Screening Unit. [(accessed on 18 March 2021)];2014 Available online: https://www.nsu.govt.nz/system/files/resources/cost-effectiveness-newborn-screening-severe-combined-immune-deficiency.pdf.
15. Van der Ploeg C.P.B., Blom M., Bredius R.G.M., van der Burg M., Schielen P.C.J.I., Verkerk P.H., Van den Akker-van Marle M.E. Cost-effectiveness of newborn screening for severe combined immunodeficiency. Eur. J. Pediatr. 2019;178:721–729.
16. Blom M., Bredius R.G.M., Jansen M.E., et al. Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J. Clin. Immunol. 2021;41:99–108.

17. Blom M., Pico-Knijnenburg I., Imholz S., Vissers L., Schulze J., Werner J., Bredius R., van der Burg M. Second tier testing to reduce the number of non-actionable secondary findings and false-positive referrals in newborn screening for severe combined immunodeficiency. J. Clin. Immunol. 2021. Online ahead of print.
18. Gold M., Siegel J., Russell L., Weinstein M. Cost-Effectiveness in Health and Medicine. Oxford University Press; New York, NY, USA: 1996.
19. Hakkaart-van Roijen L., Van der Linden N., Bouwmans C.A.M., Kanters T.A., Tan S.S. Costing Manual: Methodology of Costing Research and Reference Prices for Economic Evaluations in Healthcare. National Health Care Institute; Diemen, The Netherlands: 2015. [in Dutch: Kostenhandleiding: Methodologie van kostenonderzoek en referentieprijzen voor economische evaluaties in de gezondheidszorg]
20. Kanters T.A., Bouwmans C.A.M., van der Linden N., Tan S.S., Hakkaart-van Roijen L. Update of the Dutch manual for costing studies in health care. PLoS ONE. 2017;12:e0187477.
21. Dutch Healthcare Authority. [(accessed on 4 March 2021)]. Available online: https://zorgproducten.nza.nl/
22. Thomas C., Durand-Zaleski I., Frenkiel J., et al. Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clin. Immunol. 2019;202:33–39.
23. Van der Spek J., Groenwold R.H., van der Burg M., van Montfrans J.M. TREC Based Newborn Screening for Severe Combined Immunodeficiency Disease: A Systematic Review. J. Clin. Immunol. 2015;35:416–430.
24. Zinnige en Duurzame Zorg Raad voor Volksgezondheid en Samenleving; 2006 June. English: Fair and Sustainable Care. Council for Public Health and Society. 2006. [(accessed on 27 August 2021)]. Available online: https://www.raadrvs.nl/documenten.

SUPPLEMENTARY MATERIAL

Model parameters and their values in the model of Van der Ploeg et al. [15] and the adaptations made based on real-life data for the different screening strategies (TREC ≤ 6 copies/3.2 mm punch, TREC ≤ 10 copies/3.2 mm punch) and new screening algorithm1

Parameter	**Base case model (Van der Ploeg et al. [15])**
1. EPIDEMIOLOGICAL PARAMETERS	
Incidence of SCID	1.72/100,000 (=1/58,000 newborns)
% SCID-patients early detected without neonatal screening	20%
Incidence of non-SCID	7.1/100,000 (=1/14,000 newborns)
% non-SCID patients detected without neonatal screening	100%
Probability to survive until treatment when SCID is detected early	94%
Probability to survive after treatment when SCID is detected early	92%
Probability to survive until treatment when SCID is detected late	78%
Probability to survive after treatment when SCID is detected late	80%
Health status after transplantation (early/late detection)	Good: 80% / 50% Medium: 15% / 30% Poor: 5% / 20%
Life expectancy after transplantation (dependent on health status)	Good: 65 years (discounted:40.8 y) Medium: 40 y (30.3 y) Poor: 25 y (21.4 y)
Quality of life (utility)	Good: 0.95 Medium: 0.75 Poor: 0.5
No of children without SCID who get flow cytometry (plus visit to clinic) because of suspected SCID	10 per child with SCID without screening in place
2. SCREENING PARAMETERS	
% < cut-off TREC at first screen, i.e. retest on same sample	0.39% at <25 TREC/µl
% second heel prick	0.25%
% children with flow cytometry in total screened population	0.08%

	Adaptation by screening strategy[2]		
	TREC ≤ 6 copies/3.2 mm punch	**TREC ≤ 10 copies/3.2 mm punch**	**New screening algorithm[1]**
	-	-	-
	-	-	-
	25.2/100,000(=1/3,974 newborns)	40.1/100,000(=1/2,493 newborns)	21.2/100,000 (=1/4,710 newborns)
	-	-	-
	-		
	-		
	-		
	-		
	-		
	-		
	-		
	-		
	0.28%	0.62%	0.62%
	0.016% + 0.003% repeated first heel pricks	0.028% + 0.006% repeated first heel pricks	0.061% + 0.006% repeated first heel pricks
	0.026% referrals	0.041% referrals	0.022% referrals

Continued

Parameter	Base case model (Van der Ploeg et al. [15])
Sensitivity total screening program (SCID)	100%
Sensitivity screening program (non-SCID)	100%
Distribution non-SCID into % transient, % idiopathic and % other non-SCID	7.1% transient 2.9% idiopathic, 90.0% other non-SCID
3. COST PARAMETERS [3]	
Costs of screening test (TREC within NBS program)	TREC: € 4.71 (€4.36 +devices €0.35)
Costs of retest (duplo)	TREC: € 9.42
Costs of second heel prick	€ 29.01 (blood collection €20.30 + postage €1.60 + processing €2.40 + TREC test)
Costs of diagnostics for referred children	€ 1598 (pediatrician €102, flow cytometry (€498 incl. clinic visit), repeat flow cytometry for 2/3 of screen positives, genetic tests of €2000 for 1/3)
Costs of diagnostics in situation without screening for children with SCID or non-SCID	€ 2600 per child with SCID or non-SCID (pediatrician €102, flow cytometry (€498 incl clinic visit), genetic tests €2000)
Costs of transplantation SCID when detected early	€ 90,000
Costs of transplantation SCID when detected late	€ 205,000
Costs of treatment non-SCID per type	Transient: € 2,200 Idiopathic: € 6,200 Other: € 6,200
Costs of treatment for child with SCID which dies before transplantation	€ 135,000
Costs of treatment in remaining lifetime, dep. on health status (per year)	Good: € 26 Medium: € 18,148 Poor: € 9,713
Costs at end of life (per year, during last 5 years)	Good: € 0 Medium or poor: € 6,314 because of lung disease/ malign.

[1] Direct referral if TREC levels ≤ 2 copies/3.2 punch, and cases with TREC levels > 2 to ≤ 10 require a second heel prick after seven days.

[2] '-' means that no adaptations are made

[3] €2016 for base case model Van der Ploeg et al. [15] and €2020 for adaptations made in current study

	Adaptation by screening strategy[2]		
	TREC ≤ 6 copies/3.2 mm punch	**TREC ≤ 10 copies/3.2 mm punch**	**New screening algorithm[1]**
	56.3% sec, 9.4% idio, 21.9% syndr, 12.5% fpos	56.9% sec, 11.8% idio, 17.6% syndr, 13.7% fpos	51.3% sec, 14.7% idio, 19.3% syndr, 14.7% fpos
	€ 6.36 per sample incl. retest	€ 6.36 per sample incl. retest	€ 6.36 per sample incl. retest
	€ 79.03	€ 79.03	€ 79.03
	€7517 SCID, €1547 secondary T-cell impairment, €8561 idiopathic lymphocytopenia, €6473 T-cell impairment syndromes, € 985 false-positive		
	€7517 SCID, € 486 secondary T-cell impairment, €2250 idiopathic lymphocytopenia, €5111 T-cell impairment syndromes		
	-	-	-
	-	-	-
	-	-	-
	-	-	-
	-	-	-
	-	-	-

CHAPTER 10

Recommendations for uniform definitions used in newborn screening for severe combined immunodeficiency
A narrative review

Maartje Blom, Rolf H. Zetterström, Asbjørg Stray-Pedersen, Kimberly Gilmour, Andrew R. Gennery, Jennifer M. Puck and Mirjam van der Burg

Journal of Allergy and Clinical Immunology, 2021; 16: S0091-6749(21)01401-9.

ABSTRACT

Background

Public health newborn screening (NBS) programs continuously evolve, taking advantage of international shared learning. NBS for severe combined immunodeficiency (SCID) has recently been introduced in many countries. However, comparison of screening outcomes has been hampered by use of disparate terminology and imprecise or variable case definitions for non-SCID conditions with T-cell lymphopenia.

Objective

Standardized screening terminology could overcome a Babylonian confusion, while improved case definitions would promote international exchange of knowledge.

Methods

A systematic literature review highlighted the diverse terminology in SCID NBS programs internationally. While, as expected, individual screening strategies and tests were tailored to each program, we found uniform terminology to be lacking in definitions of disease targets, sensitivity and specificity required for comparisons across programs.

Results

Our recommendations reflect current evidence from literature and existing guidelines coupled with opinion of experts in public health screening and immunology. Terminologies were aligned. The distinction between actionable and non-actionable T-cell lymphopenia among non-SCID cases was clarified, the former being infants with T-cell lymphopenia who could benefit from interventions such as protection from infections, antibiotic prophylaxis, and live-attenuated vaccine avoidance.

Conclusions

By bringing together the previously unconnected public health screening community and clinical immunology community, our SCID NBS deliberations bridged the gaps in language and perspective between these disciplines. We propose that international specialists in each disorder for which NBS is performed join forces to hone their definitions and recommend uniform registration of outcomes of NBS. Standardization of terminology will promote international exchange of knowledge and optimize each phase of NBS and follow-up care, advancing health outcomes for children worldwide.

INTRODUCTION

In the past decade, newborn screening (NBS) for severe combined immunodeficiency (SCID), the most profound inborn error of immunity (IEI), has been introduced in many screening programs worldwide [1, 2]. Prompt clinical intervention with hematopoietic stem cell transplantation (HSCT) or gene therapy is required to prevent morbidity and early mortality for these patients [3, 4]. SCID is the first immune disorder to be accepted for population-based screening, and implementation has provided important clinical benefits for affected infants as well as lessons for public health programs, immunologists and pediatricians.

NBS for SCID is based on quantification of the molecular biomarker T-cell receptor excision circle (TREC), a byproduct of the normal recombination of the T-cell receptor genes as thymocytes differentiate into mature T-cells [5]. TRECs are quantitated by PCR in DNA isolated from infant dried blood spots (DBS). Infants with SCID lack T-cells, and consequently the absence of TRECs in their DBS identifies SCID with remarkable sensitivity [6]. However, other non-SCID conditions associated with T-cell lymphopenia in the neonatal period are also identified as having fewer TRECs than normal, leading to reduced specificity that must be addressed by each individual SCID NBS program [7, 8]. In NBS for SCID, case definitions for actionable T-cell lymphopenia, non-actionable T-cell lymphopenia and secondary findings have not previously been clearly defined.

Public health programs have the responsibility to continuously optimize NBS for their stakeholders. International shared learning will expedite effective implementation of SCID screening for all infants. However, when sharing experiences, a challenging hurdle has arisen. Comparison of screening algorithms, cut-off values and referral policies, as well as uniform registration of cases with abnormal screening results, have to date been hampered by differing terminology between NBS programs. Simply said 'it's a mess,' and there is need for standardization of screening terminology to avoid a Babylonian confusion.

Our group, representing specialists with direct experience in screening, clinical immunology and pediatrics, has used SCID to illustrate the divergence of screening terms used in NBS programs for SCID worldwide. With the aid of a systematic literature search and existing guidelines, we considered the range of terminologies for reporting NBS test results, screening strategies, case definitions and clinical outcomes. Most importantly, we suggest uniform definitions for SCID screening test outcomes and diagnostic follow-through to be used in scientific publications

and registries. These recommendations are designed to aid all screening programs, uniting the SCID screening community with the clinical immunology community, while suggesting a critical revaluation of case definitions used for other screened disorders as well as SCID.

METHODS

Systematic review

A systematic review was conducted on NBS for SCID and case definitions used in pilot studies and population based screening. An electronic search was performed on MEDLINE (PubMed), EMBASE (excluding MEDLINE), Cochrane library and Scopus databases. The search strategy is shown in the Supplementary Material. The study selection flow diagram is shown in Figure 1. The eligibility criteria, study selection, data extraction and quality assessment are specified in the Supplementary Material.

Guidelines and panel

Existing guidelines of the European Society for Immunodeficiencies (ESID) [9], the Association of Public Health Laboratories (APHL) [10], the Clinical & Laboratory Standards Institute (CLSI) [11], Primary Immune Deficiency Treatment Consortium experience (PIDTC) [12], Clinical Immunology Society (CIS), Immune Deficiency Foundation (IDF) [13] and International Union of Immunological Societies (IUIS) [14, 15] were evaluated and considered when formulating recommendations. Meetings were held with leading experts in the field of NBS for SCID, IEI, immunological diagnostics, genetics and stem cell transplantation. The panel, consisting of seven members from five different countries, came together after a virtual meeting on NBS for SCID organized by the International Society for Neonatal Screening (ISNS) and the UK Newborn Screening Laboratory Network (UKNSLN). Each member brought his/her own expertise and experience in NBS for SCID, and together the group formulated consensus-based recommendations reflecting all currently available evidence.

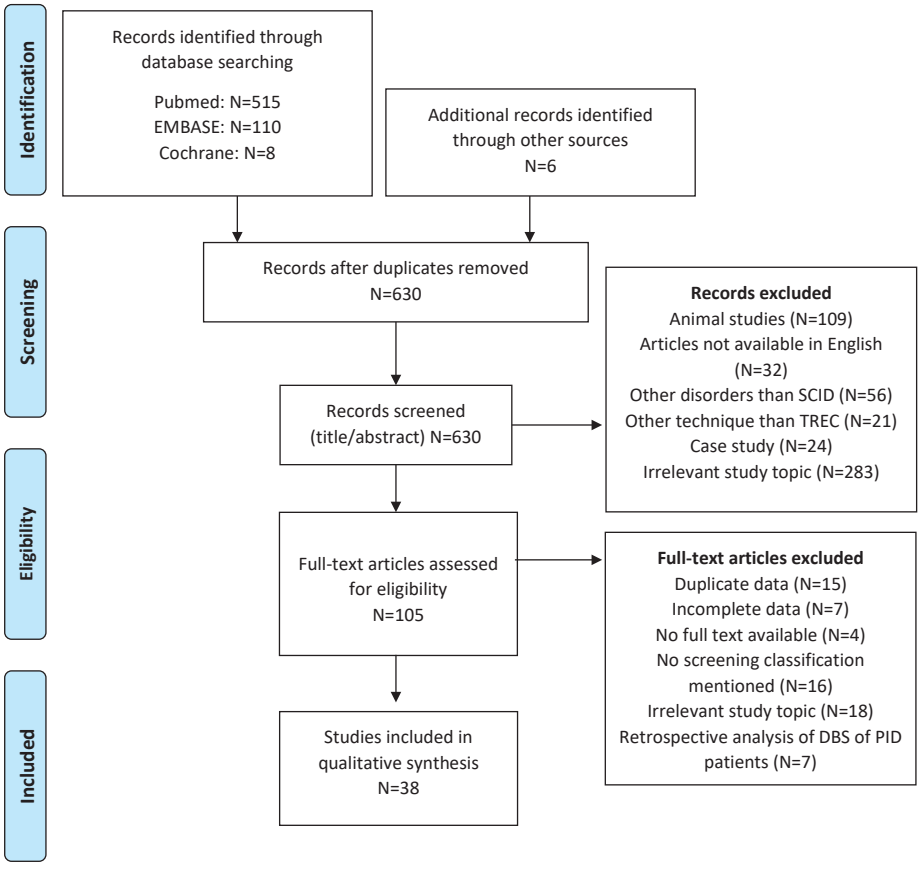

Figure 1. Flow diagram used for article selection in the systematic review of definitions used in NBS for SCID. Search performed on 16 February 2021.

OBSERVATIONS

NBS programs use different definitions in literature

Our search resulted in 630 unique records. By checking the reference lists of selected articles, we included six additional articles. After screening abstracts and titles, 38 articles were included in the qualitive analysis (Figure 1). Four overview articles [16-19], 11 population based studies [20-30], 20 pilot studies [31-50] and three studies including both pilot and population data [51-53] were included. The number of screened newborns ranged from N = 141 in Korea [37] to N = 3 252 156 in California [22], with varying referral and retest rates between screening programs. Study characteristics are further specified in Table S1.

Definitions of screening results used in studies on NBS for SCID

Definitions predominantly used to describe NBS test results were *negative* or *normal* (TRECs above cut-off) versus *positive* or *abnormal* (TRECs below cut-off) (Figure 2A). Some programs distinguished between *positive* and *urgent positiv*e test results, with the lowest TREC -levels requiring more rapid follow-up actions [27, 31]. One study used the opposite terminology defining TREC positive as present TRECs (Cp value <37.0) and TREC *negative* as low/absent TRECs (Cp value >39.0) [37]. Users of the EnLite TREC assay (PerkinElmer) often included *presumptive positive* to specify that TRECs were below cut-off after repeated analysis on the same NBS card in duplicate [16, 45, 48, 52]. *Inconclusive* was the predominant terminology used for failure of internal control amplification, but *indeterminate* [16], *incomplete* [22, 27, 31] and *unsatisfactory* [50] were also described (Figure 2A; Table S2).

Definition of variables in the screening algorithm used in studies on NBS for SCID

There is a range of terms used to describe certain actions in screening algorithms employed at public health screening laboratories. *Retesting* was most commonly used to indicate repeated TREC analysis; most NBS program perform this analysis on the same NBS card either re-using the original DNA extract and/or DNA from a new punch from the same card, while other programs use the term *retest* when requesting a new NBS card from the infant (Figure 2B; Table S3). Other terms used for *retesting* are *repeat(ed) testing*, *reanalysis*, *duplicate/second analysis*, *re-run*, *second punch analysi*s and *second run*. Requesting a second NBS card was usually more diversely described by terms such as *second (NBS/DBS) sampling, second Guthrie card, new sample/NBS card, re-sampling, redraw, second heel prick, second DBS request, repeat NBS/DBS (specimen), repeat sampling* etc. To indicate that a newborn with low TREC levels was evaluated by a pediatrician or immunologist with follow-up diagnostics, *referral* was primary used (Figure 2B). In contrast, some

programs included the term *recall* or *call back,* which could mean both an infant recalled for a new DBS sample by a nurse or pediatrician, as well as an infant sent to receive a clinical evaluation, flow cytometric diagnostics, and genetic testing [41, 42].

Classification of (case) definitions and outcomes after follow-up used in studies on NBS for SCID

Classification of 'diagnoses' or outcomes after an abnormal SCID screening result differed greatly between NBS programs (Table S4). Some programs used their own criteria to define SCID, while others used criteria from existing guidelines such as those published by the PIDTC [12]. In some, but not all programs, SCID was subclassified into typical, leaky/atypical and Omenn syndrome. Non-SCID T-cell lymphopenia was generally divided into (i) syndromes that include variable T-cell impairment (or non-SCID T-cell lymphopenia due to syndromes/ syndromic patients); (ii) secondary T-cell lymphopenia (or transient T-cell lymphopenia due to a non-immunologic neonatal condition); and (iii) idiopathic T-cell lymphopenia (in some case referred to as *variant SCID*). Premature birth alone was mentioned as a separate outcome category in 15 out of 38 studies, but otherwise was included with secondary T-cell lymphopenia. *False-positive* referrals were mentioned in 12 studies, but exact descriptions of the term varied. Finally, some publications listed the status of newborns (e.g. flow cytometry pending or lost to follow-up) or all diagnoses without classification, while five pilot studies were unable to classify newborns with low TRECs the due to anonymized inclusion and no clinical follow-up.

Definitions of premature infants used in studies on NBS for SCID

The majority of the included studies defined prematurity as a gestational age < 37 weeks. Some NBS programs discriminated between moderate/very/extremely preterm [31] or included low birth weight (≤ 2500 grams) as an additional parameter [32, 45] In other reports, prematurity was mentioned, but not further specified, or not reported at all. Many programs have tried to limit their number of referrals by including adjustments in their screening algorithm for preterm infants with low TREC levels. Countries are requesting second NBS cards when preterm newborns reach a certain gestational age, monitoring preterm infants with serial NBS specimens, or using a lower TREC cut-off value for premature infants (Table S5).

Figure 2A

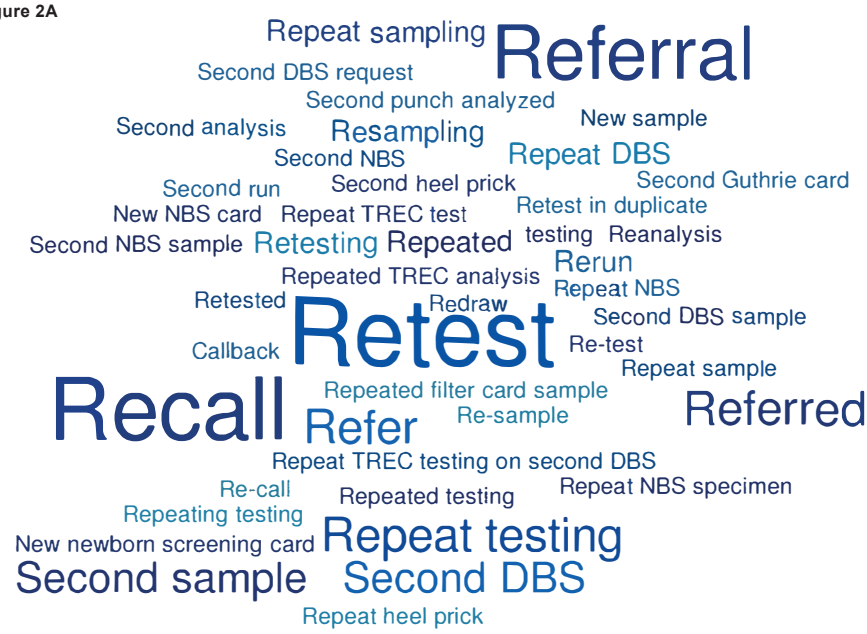

Figure 2B

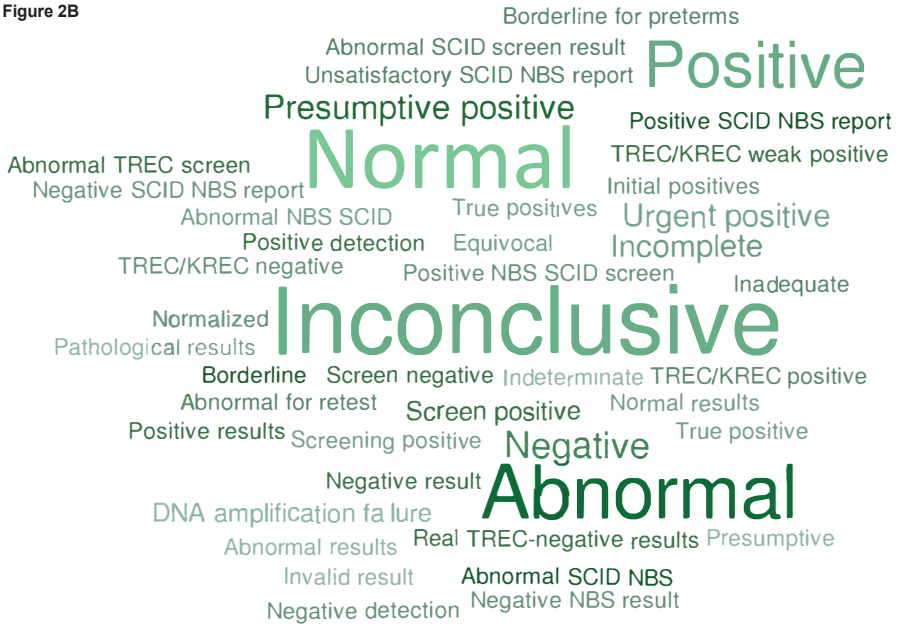

Figure 2 A. Different terminology used for screening results in studies on NBS for SCID. Wordcloud based on Table E2. **B**. Different terminology used for variables in screening algorithms studies on NBS for SCID. Wordcloud based on Table E3.

Guidelines use different definitions

Different guidelines are available to classify NBS SCID outcomes or to help clinicians in diagnosing IEI based on clinical, biological and genetic features. In addition to published NBS studies, the new uniform definitions for SCID NBS must take immunologic diagnostic criteria into account to assure that terminology and classifications apply seamlessly for all phases of the screening program from initial DBS testing through diagnosis and outcomes after follow-up.

ESID has developed working definitions for clinical diagnosis of IEI [9] that can help clinicians with a clinically probable diagnosis of a symptomatic individual being evaluated prior to genetic testing. The criteria include invasive or opportunistic infections or other symptoms, a positive family history, manifestations of disease early in life, and exclusion of HIV; there are also T-cell specific laboratory results. ESID provides suggestions for alternative diagnosis if the criteria are not completely fulfilled [9].

The APHL has provided case definition tables for all disorders included in NBS programs, including SCID [10]. The SCID definitions were created by a panel of experts between 2011 and 2013, updated in 2018. A distinction was made between the primary target of NBS (typical SCID, leaky SCID and Omenn syndrome) and secondary targets (syndromes with variable immune defects with some cases having significantly low T-cell numbers, secondary T-cell lymphopenia and idiopathic T-cell lymphopenia). The primary target diagnoses are classified as either definitive, probable, or possible or uncertain based on CD3 T cells/µL, proliferation to phytohemagglutinin (PHA), maternal engraftment, molecular testing and clinical presentation. For non-SCID T-lymphopenic conditions, maternal engraftment would be absent, T-cells might be largely naïve (bearing the surface marker CD45RA or equivalent) and PHA proliferation would usually be normal [10].

The CLSI provided a guideline for NBS for SCID by measurement of TRECs in 2013, including a chapter on terminology and definitions (NBS06-A) [11]. A distinction was made between (1) typical SCID, (2) leaky SCID and Omenn syndrome, (3) variant SCID, (4) syndromes with primary T-cell lymphopenia, (5) secondary T-cell lymphopenia not due to prematurity alone and (6) preterm infants. Diagnoses in these categories were further explained in the Appendix of the CLSI document. CLSI also provided definitions for other screening parameters such as false-positives/negatives, screen positive/negative results and retests [11]. A new version of the CLSI guideline is currently being developed.

In 2014, the PIDTC developed a uniform set of criteria for diagnosing SCID and related disorders by an expert group who have seen substantial numbers of SCID cases over many years [12]. SCID patents (N = 285) were retrospectively assigned to one of three strata: *A.* Typical SCID, *B.* Leaky SCID, Omenn SCID and Reticular Dysgenesis and *C.* SCID with non-HSCT treatments. Using strict eligibility criteria [12], 86% of patients with SCID or SCID-related conditions could be assigned to one of the established strata. Lack of critical laboratory information led to difficulties in dealing with the remaining 14% of the patients. The experts acknowledged that the criteria might evolve over time and highlighted the increasing role of genotyping in establishing diagnosis, particularly in the setting of NBS.

The CIS refers to the diagnostic and clinical care guidelines for PIDs from the IDF [13] and the classification of IUIS [14]. IDF is a national patient organization that developed these guidelines in partnership with expert immunologists to enhance earlier diagnosis. The IDF distinguishes SCID with reticular dysgenesis, SCID with low T- and B-cell numbers, SCID with low or normal B-cell numbers and other combined immunodeficiencies. In addition, DiGeorge syndrome, ataxia telangiectasia and Wiskott-Aldrich syndrome are also listed under cellular or combined immunodeficiencies.

The IUIS expert committee has published and updated biannually a genotypic and phenotypic classification of all IEIs [14, 15]. This classification is organized into tables, each of which attempts to group IEIs sharing a given pathogenesis and immunologic features . Clinical and laboratory results are used for the diagnostic algorithm and phenotypical classification. (Severe) combined immunodeficiencies affecting both cellular and humoral immunity already include > 50 different disorders caused by mutations in 58 genes. T-cell lymphopenia in SCID is defined by $CD3^+$ T cells < 300/μL [14, 15]. The IUIS gene lists have grown and become more complex as the discovery of novel IEI disorders has been occurring at an impressive rate. In addition, the clinical spectrum has become broader for many conditions as more patients are observed [54].

SUGGESTIONS FROM OUT GROUPS OF SPECIALISTS

The authors have aimed to underline the gaps in language and perspective between the NBS community and the field of clinical (diagnostic) immunology. Immunologists have already developed international nomenclature to describe cell phenotypes, enabling easy cross-border communication. A similar language is required for outcomes of NBS SCID to enable comparison of NBS programs. International shared learning between public health programs and immunologists will expedite effective implementation

of SCID screening for all infants. There is need to bring these disciplines together by creating shared case definitions to exchange information via uniform registration of screening outcomes in scientific publications and registries to optimize and improve NBS programs worldwide.

Constraints of individual programs: harmonization of screening strategies is not required, but uniform registration of screening outcomes

The authors acknowledge that there are constraints of individual programs, and certain terms have been incorporated in NBS for many years. NBS programs use a variety of test methods, cut-off values and screening algorithms to balance a high sensitivity, detecting all SCID patients, while preventing high referral rates in their particular populations. Some programs have included the request of a second NBS card in their screening algorithm, while others have included second-tier tests such as next generation sequencing (NGS) [51]. In addition, other test methods such as tandem mass spectrometry i.e. for ADA or PNP deficiency have been proposed [55, 56]. There is no need to harmonize individual screening strategies; but to avoid confusion, the authors want to recommend uniform designations for screening outcomes independent of how they are generated. NBS programs can use their own definitions in practice, but are encouraged to conform to uniform terminology when publishing program outcomes internationally.

Considerations in defining screening terminology

The systematic literature review highlighted the diversity of terminology used in NBS programs. Clear recommendations without ambiguity are required for clinicians, public health specialists and other NBS stakeholders such as policy makers and parents. *Positive* and *negative* are commonly used terms in NBS, but definitions vary between programs. 'TREC positive' could imply the presence of TRECs, but the term *positive* is also broadly used for a screen with TRECs below cut-off. In addition, families can interpret a positive test result as 'positive' or good news. *Abnormal* and *normal* are non-specific terms that can have negative connotations. Labeling an infant as abnormal causes parental anxiety, while the term normal excludes the fact that newborns can have serious disorders not screened for. The terms *within normal range* or *outside normal range* might be preferred, but ranges are not applicable to SCID NBS because only TRECs below a certain cut-off value are important. The authors therefore recommend the terms *abnormal value* and *normal value* to describe TREC screening results (Recommendations Figure 3). *Incomplete* is recommended if further action is required due to DNA amplification failure.

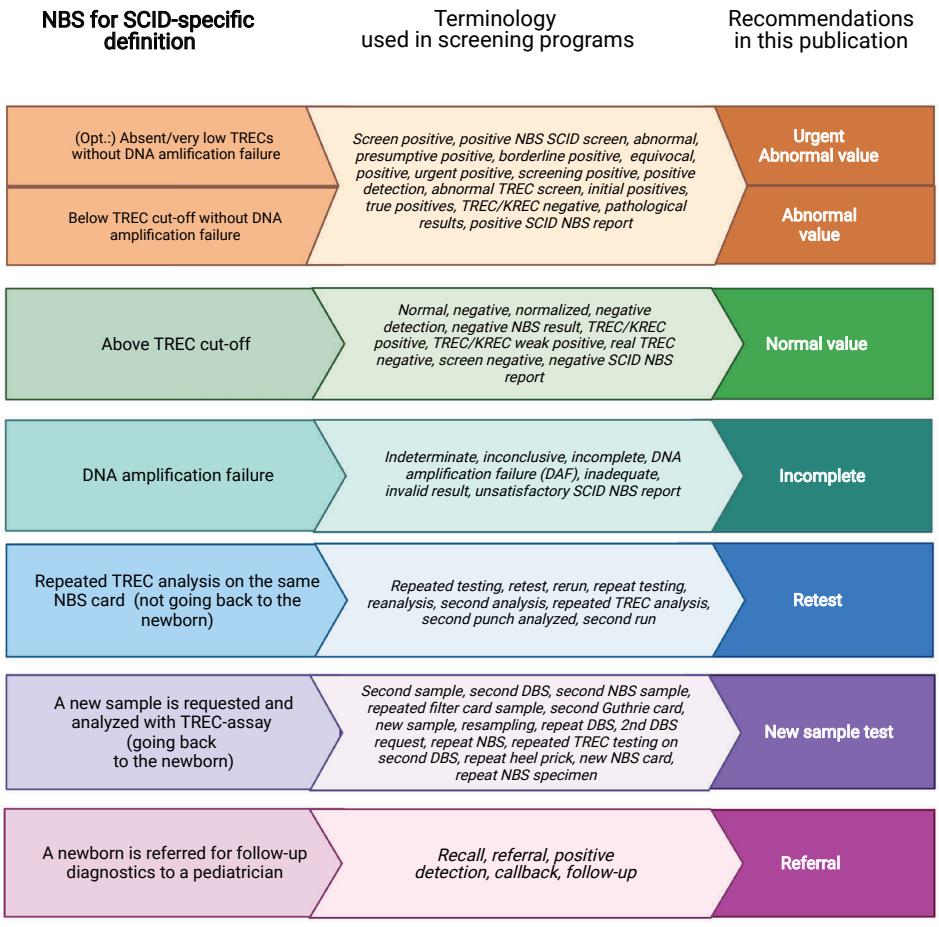

Figure 3. Recommendations on definitions of screening terminology.

For screening algorithm outcomes, the authors agree with the term *retest* which is commonly used in literature. However, it should be specified that retesting is TREC analysis of the same NBS card (not going back to the newborn for a new card). If TREC analysis is repeated on a new NBS card, the authors feel that the term *new sample test* is best. Second NBS card/sample is not completely correct as some programs are requesting a routine second NBS card for other disorders, such as congenital hypothyroidism, and this new sample to resolve SCID screening could be the third NBS card. It is important to highlight when a new sample is taken from the newborn as repeated sampling is not without anxiety and emotional insecurity for parents and additional distress for the newborn. Finally, the authors prefer the term *referral* (meaning sending for specialist evaluation) over *recall*, as recall is differently used across programs (Recommendations Figure 3).

Considerations in defining diagnostic outcomes after an abnormal value screening test
In addition to unique screening strategies, screening programs for SCID also differ in diagnostic approaches and follow-up of newborns with low TREC levels. Existing guidelines describing diagnostic criteria for SCID and other immunodeficiencies are of great aid to clinicians in facilitating diagnosis of these conditions worldwide. The authors therefore recommend to define SCID according to the widely used PIDTC guidelines, which also allow subcategorization into leaky SCID and Omenn syndrome [12]. Even though diagnostic guidelines help immunologists with a prompt and consistent approach to a definitive diagnosis, the translation to the NBS community, which should also include definitions of non-SCID T-cell lymphopenic conditions, is lacking. Thus we recommend to subdivide non-SCID T-cell lymphopenia into three categories: (i) *syndromes that can be associated with T-cell impairment*, (ii) *reversible conditions with T-cell impairment that resolves upon treatment of the underlying cause*, and (iii) *idiopathic T-cell lymphopenia*. The term *variant SCID*, originally considered analogous to variant forms of inborn errors of metabolism, should not be used as it does not describe any specific group of immunodeficient patients recognized by immunologists; while the term has been applied in the screening phase of SCID NBS programs, it has no counterpart in the diagnostic setting of immunology specialty care.

Preterms and/or newborns with low birth weight should be a separate category, only including preterm infants (gestational age < 37 weeks) and/or newborns with low birth weight (< 2500 grams) who have low T-cells without other preexisting conditions associated with T-cell lymphopenia. The term *false-positive* can lead to confusion as some NBS programs define all referrals, with a diagnosis other than the disorder primary screened for, SCID, as false-positives referrals. In addition, T-cells may have been low at birth but normalized in the first week up to referral, reflecting a true transient T-cell lymphopenia. The term *normal T-cell subsets* is therefore better suited to avoid confusion. Finally, a subcategory was added to address *inconclusive* classification for newborns who have died prior to follow-up diagnostics or who are lost to follow-up without referral. Our recommendations will help with systematic registration of referred newborns and allow evaluation of NBS programs in a broader international perspective (Recommendations Figure 4).

Actionable T-cell lymphopenia versus non-actionable T-cell lymphopenia and secondary findings
An important aspect of TREC screening for SCID is the wide spectrum of different disorders that are detected by this single parameter. The TREC assay for SCID confers a high sensitivity compared to many established NBS disorders [57]. In contrast, if one includes only SCID as the primary target of screening, the positive predictive

value (PPV) is quite low as compared to some other screened disorders. NBS for SCID by quantification of TRECs identifies a range of neonatal conditions and disorders associated with T-cell lymphopenia in the neonatal period, which some programs define as secondary or incidental findings. NBS with TREC testing correlates with having recently formed T-cells in peripheral blood; therefore one could argue that in TREC-based screening primary targets should include all serious, actionable T-cell deficiencies. From the clinical immunologist's point of view, any newborn with a disorder in which prompt intervention can prevent morbidity and mortality should be flagged in a NBS program. NBS programs tend to focus on a primary target, although secondary targets/findings might be defined if there is a clear health benefit for the child. Policies differ between countries and individual screening programs in classifying severe T-cell deficiencies as primary or secondary targets (or findings) of NBS for SCID, and each NBS program will need to reach its own decision in this multifaceted discussion.

The authors feel that a distinction should be made between *actionable* and *non-actionable* T-cell lymphopenia and secondary findings, although it can be challenging to make clear statements about actionability. From a parental perspective, the benefit for actionable disorders lies in the possibility of managing the disease upon recognizing it early in an infant's life, thus improving health and social outcomes [58]. Even in the absence of a cure, early diagnosis may lead to strategies resulting in health benefits such as prevention of comorbidities, facilitated access to social care and support and improved quality of life. Parents also address the avoidance of a diagnostic odyssey and the option to make informed reproductive choices as clear health benefits, but the authors will limit their definition of actionability to the management of the individual affected with the condition. The term *actionable* indicates that an urgent (early) intervention is required by a specialist and that the intervention results in a demonstrated improvement in outcome. Neonates with profound T-cell lymphopenia, not meeting all criteria for SCID but eligible for HSCT, would undisputedly be classified as an actionable finding. The same would be applicable for patients with complete 22q11.2 deletion syndrome (DiGeorge syndrome), CHARGE syndrome, athymic FOXN1 deficiency or PAX1 deficiency, all of which are indications for thymus transplantation [59-62]. Pediatric-immunologists propose that cases of significant T-cell lymphopenia that might benefit from antibiotic prophylaxis, protective isolation, or avoiding live-attenuated vaccines should also be deemed actionable [63, 64] For these cases, one could argue that the term *actionable* depends on absolute T-cell number and the duration of the T-cell defect. The term *actionable* is more suitable than the term *treatable*, as withholding live-attenuated vaccines is an important early intervention leading to improved outcomes, given that vaccine-strain organisms can cause serious infections in individuals with T-cell defects.

Suggested definition in NBS for SCID	Terminology used in screening programs	Recommendations in this publication
SCID is defined according to the PIDTC classification [12]	*SCID, typical/classical SCID, leaky SCID, atypical SCID, Omenn syndrome, radiosensitive SCID*	**SCID**
Cases with low T-cell numbers on confirmatory testing, but that did not meet the criteria for SCID	*Non-SCID & non-syndrome T-cell lymphopenia, non-SCID T cell lymphopenia, non-SCID TCL, abnormal flow cytometry*	**Non-SCID T-cell lymphopenia**
Newborns with a recognized genetic syndrome that includes low T-cell numbers within its spectrum of clinical findings	*Syndrome chromosomal abnormalities, non-SCID T cell lymphopenia due to syndrome, T-cell impairment syndromes, congenital syndrome, syndromic patients, conditions with primary TCL*	**a. Syndromes with T-cell impairment**
Newborns with low T-cell numbers attributed to other medical conditions without an intrinsic defect in the production of T-cells e.g. heart defects, congenital anomalies, maternal immunosuppressant medication	*Secondary T cell lymphopenia, transient lymphopenia, secondary T-cell impairment, T-cell loss or destruction,, secondary, lymphopenia due to secondary cause, T-cell loss, reversible TCL, - extravasation of T -cells outside vascular space*	**b. Reversible conditions with T-cell impairment**
Newborns with low T-cell numbers in which an underlying condition could not be determined, even after immunologic and comprehensive genetic evaluation	*Unknown etiology, variant SCID, T- cell lymphopenia of unknown reason, idiopathic T-cell lymphopenia, unspecified T-cell lymphopenia,*	**c. Idiopathic T-cell lymphopenia**
Newborns with gestational age <37 weeks and/or birth weight <2500 grams with low T-cell numbers and no other clinical explanation for low TRECs.	*Prematurity, premature, premature infants, TCL and premature birth alone, preterm birth alone, preterm*	**Preterm and/or low birth weight alone**
Newborns with normal for age T-cell numbers and normal naïve CD4+ cells determined with flow cytometry	*False positives, not SCID, normal flow cytometry, spontaneously normalized, negative, normal CBC and flow cytometry, normal*	**Normal T-cell subsets**
No conclusion on classification possible e.g. deceased newborns or lost to follow-up	*Visit with no flow cytometry/deaths with no flow cytometry, unspecified, declined resampling, died prior to resampling, declined follow-up, pending further evaluation, expired, no diagnosis, lost to follow-up, parental refusal*	**Inconclusive**

Figure 4. Recommendation on classification of diagnostic outcomes after an abnormal value screening test

Non-actionable secondary findings may be relevant prognostically, but either effective treatments are not available or health benefits from early diagnosis are limited or uncertain. The aim of population based screening is to prevent morbidity or mortality from the targeted disorders through earlier treatment and with limited harm to unaffected infants. Non-actionable secondary findings and referrals of infants with normal lymphocyte numbers by flow cytometry raise concerns about the harm-benefit ratio of screening, and public health programs justifiably strive to prevent referral of these cases [65].

Defining targets for other conditions for which NBS is taking place

By better defining disease targets in a NBS program, parameters such as sensitivity/specificity and PPV can be reported and compared across programs, improving existing programs, but also aiding in policy with regard to pilot studies. NBS is a multi-faceted system, and pilot studies provide the opportunity to consider addition of new disorders without disrupting the program. However, for smaller countries and in the case of rare diseases, pilot studies would require many years to generate data about sensitivity or PPV. If screening outcomes can be uniformly interpreted across borders, smaller countries might rely on test validation in screening laboratories and limit pilot studies to unique aspects of their locale. At this point, knowledge gained by other countries is not optimally used. If we would do so, swift implementation of new disorders could be achieved, saving time and money and leading to the most health gain for affected newborns. We suggest that international experts from each discipline included in NBS (e.g. inborn errors of metabolism, congenital hypothyroidism, cystic fibrosis, hemoglobinopathies, etc.) join forces to discuss the target definitions and to provide their own recommendations for uniform registration of outcomes.

The importance of uniform registration of screening outcomes

Public health programs have a responsibility towards their stakeholders to continuously improve and optimize their NBS programs. Opportunities for improvement can be identified only if outcomes can be compared to unscreened populations or to other NBS programs. For international shared learning, harmonized registration of screening terminology and case definitions is a prerequisite. Evaluation of the screening terminology should be an ongoing process for continuous optimization of NBS programs. Trust in population screening programs is one of the key elements for parents when participating in NBS. By continuously optimizing laboratory algorithms and screening programs, increasing the PPV, one can limit the risk of unnecessary referrals that are associated with high emotional impact for parents and invasive diagnostic testing for the child [32, 65, 66]. More importantly, a NBS program should aim to achieve the highest sensitivity, avoiding missing affected children in the direct

health interest of the child. In addition, public health programs have a responsibility towards the society as a whole, as screening requires resources, and referrals are associated with high diagnostic costs. Cost-effectiveness analyses that are needed to justify NBS programs can be well-executed only if screening outcomes are registered in a uniform manner.

CONCLUSION

Our recommendations reflect currently available evidence including a systematic literature review and existing guidelines coupled with expert opinion. By bringing two audiences together, the NBS community and the clinical immunology community, our guidelines will unite the field by bridging the gaps in language and perspective between these disciplines. Standardization of terminology and uniform registration of screening outcomes will promote international exchange of knowledge and improve NBS programs and follow-up care resulting in better health outcomes for children worldwide.

REFERENCES

1. King, J., J.F. Ludvigsson, and L. Hammarström, Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future. International Journal of Neonatal Screening, 2017. 3(3): p. 19.
2. van der Burg, M., et al., Universal Newborn Screening for Severe Combined Immunodeficiency (SCID). Front Pediatr, 2019. 7: p. 373.
3. Pai, S.Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med, 2014. 371(5): p. 434-46.
4. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
5. Hazenberg, M.D., et al., T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J Mol Med (Berl), 2001. 79(11): p. 631-40.
6. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol, 2005. 115(2): p. 391-8.
7. Buchbinder, D., et al., When Screening for Severe Combined Immunodeficiency (SCID) with T Cell Receptor Excision Circles Is Not SCID: a Case-Based Review. Journal of Clinical Immunology, 2021. 41(2): p. 294-302.
8. Mauracher, A.A., et al., Causes of low neonatal T-cell receptor excision circles: A systematic review. J Allergy Clin Immunol Pract, 2017. 5(5): p. 1457-1460.e22.
9. ESID_Registry. Working definitions for clinical diagnosis of PID. 2019 [cited 2021 2 June]; Available from: https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria.
10. APHL. Case Definitions for Newborn Screening. 2018 [cited 2021 2 June]; Available from: https://www.newsteps.org/data-resources/case-definitions.
11. CLSI, Newborn Bloot Spot Screening for Severe Combined Immunodeficiency by Measurement of T-cell receptor Excision Circles Approved Guideline. 2013. p. ISBN 1-56238-871-1.
12. Shearer, W.T., et al., Establishing diagnostic criteria for severe combined immunodeficiency disease (SCID), leaky SCID, and Omenn syndrome: The Primary Immune Deficiency Treatment Consortium experience. Journal of Allergy and Clinical Immunology, 2014. 133(4): p. 1092-1098.
13. IDF. Immune Deficiency Foundation Diagnostic & Clinical Care Guidelines for Primary Immunodeficiency Diseases. 2015 [cited 2021 2 June]; Available from: https://primaryimmune.org/publication/healthcare-professionals/idf-diagnostic-clinical-care-guidelines-primary.
14. Bousfiha, A., et al., Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. J Clin Immunol, 2020.
15. Tangye, S.G., et al., Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol, 2020. 40(1): p. 24-64.
16. Richards, S., et al., Diagnosis and management of severe combined immunodeficiency in Australia and New Zealand. Journal of Paediatrics and Child Health, 2020. 56(10): p. 1508-1513.

17. Kobrynski, L.J., Identification of non–severe combined immune deficiency T-cell lymphopenia at newborn screening for severe combined immune deficiency. Annals of Allergy, Asthma & Immunology, 2019. 123(5): p. 424-427.
18. Routes, J. and J. Verbsky, Newborn Screening for Severe Combined Immunodeficiency. Current Allergy and Asthma Reports, 2018. 18(6): p. 34.
19. Madkaikar, M., J. Aluri, and S. Gupta, Guidelines for Screening, Early Diagnosis and Management of Severe Combined Immunodeficiency (SCID) in India. The Indian Journal of Pediatrics, 2016. 83(5): p. 455-462.
20. Thorsen, J., et al., Newborn Screening for Severe Combined Immunodeficiency: 10-Year Experience at a Single Referral Center (2009-2018). J Clin Immunol, 2021.
21. Argudo-Ramírez, A., et al., First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain). Front Immunol, 2019. 10: p. 2406.
22. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2).
23. Rechavi, E., et al., First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol, 2017. 8: p. 1448.
24. Rechavi, E., et al., Newborn Screening for Severe Combined Immunodeficiency in Israel. International Journal of Neonatal Screening, 2017. 3(2): p. 13.
25. Chien, Y.-H., et al., Newborn Screening for Severe Combined Immunodeficiency in Taiwan. International Journal of Neonatal Screening, 2017. 3(3): p. 16.
26. Vogel, B.H., et al., Newborn screening for SCID in New York State: experience from the first two years. J Clin Immunol, 2014. 34(3): p. 289-303.
27. Kwan, A., et al., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia in California: results of the first 2 years. J Allergy Clin Immunol, 2013. 132(1): p. 140-50.
28. Verbsky, J.W., et al., Newborn screening for severe combined immunodeficiency; the Wisconsin experience (2008-2011). J Clin Immunol, 2012. 32(1): p. 82-8.
29. Baker, M.W., et al., Implementing routine testing for severe combined immunodeficiency within Wisconsin's newborn screening program. Public Health Rep, 2010. 125 Suppl 2(Suppl 2): p. 88-95.
30. Routes, J.M., et al., Statewide newborn screening for severe T-cell lymphopenia. Jama, 2009. 302(22): p. 2465-70.
31. Giżewska, M., et al., Newborn Screening for SCID and Other Severe Primary Immunodeficiency in the Polish-German Transborder Area: Experience From the First 14 Months of Collaboration. Front Immunol, 2020. 11: p. 1948.
32. Blom, M., et al., Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J Clin Immunol, 2021. 41(1): p. 99-108.
33. Thomas, C., et al., Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clinical Immunology, 2019. 202: p. 33-39.
34. Audrain, M.A.P., et al., Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). Journal of Clinical Immunology, 2018. 38(7): p. 778-786.
35. Nourizadeh, M., et al., Newborn screening using TREC/KREC assay for severe T and B cell lymphopenia in Iran. Scand J Immunol, 2018: p. e12699.

36. Al-Mousa, H., et al., High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. Front Immunol, 2018. 9: p. 782.
37. Son, S., et al., The First Newborn Screening Study of T-Cell Receptor Excision Circle and κ-Deleting Recombination Excision Circle for Severe Combined Immunodeficiency in Korea: A Pilot Study. Pediatr Infect Vaccine, 2017. 24(3): p. 134-140.
38. Kanegae, M.P.P., et al., Newborn Screening for Severe Combined Immunodeficiencies using TRECs and KRECs: Second Pilot Study in Brazil. Rev Paul Pediatr, 2017. 35(1): p. 25-32.
39. Kanegae, M.P.P., et al., Neonatal screening for severe combined immunodeficiency in Brazil. Jornal de Pediatria, 2016. 92(4): p. 374-380.
40. Tagliaferri, L., et al., Newborn screening for severe combined immunodeficiency using a novel and simplified method to measure T-cell excision circles (TREC). Clinical Immunology, 2017. 175: p. 51-55.
41. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. 37(1): p. 51-60.
42. Zetterström, R.H., et al., Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden. International Journal of Neonatal Screening, 2017. 3(2): p. 11.
43. De Felipe, B., et al., Newborn Screening for Primary T- and B-Cell Immune Deficiencies—A Prospective Study in Andalucía. International Journal of Neonatal Screening, 2017. 3(4): p. 27.
44. de Felipe, B., et al., Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol, 2016. 27(1): p. 70-7.
45. Blom, M., et al., An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. Clin Immunol, 2017. 180: p. 106-110.
46. Chien, Y.H., et al., Incidence of severe combined immunodeficiency through newborn screening in a Chinese population. J Formos Med Assoc, 2015. 114(1): p. 12-6.
47. Audrain, M., et al., Evaluation of the T-cell receptor excision circle assay performances for severe combined immunodeficiency neonatal screening on Guthrie cards in a French single centre study. Clin Immunol, 2014. 150(2): p. 137-9.
48. Adams, S.P., et al., Screening of neonatal UK dried blood spots using a duplex TREC screening assay. J Clin Immunol, 2014. 34(3): p. 323-30.
49. Borte, S., et al., Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood, 2012. 119(11): p. 2552-5.
50. Comeau, A.M., et al., Guidelines for implementation of population-based newborn screening for severe combined immunodeficiency. Journal of Inherited Metabolic Disease, 2010. 33(S2): p. 273-281.
51. Strand, J., et al., Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol, 2020. 11: p. 1417.
52. Kwan, A., et al., Successful newborn screening for SCID in the Navajo Nation. Clin Immunol, 2015. 158(1): p. 29-34.
53. Kwan, A., et al., Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama, 2014. 312(7): p. 729-38.
54. Tangye, S.G., et al., The Ever-Increasing Array of Novel Inborn Errors of Immunity: an Interim Update by the IUIS Committee. J Clin Immunol, 2021. 41(3): p. 666-679.

55. la Marca, G., et al., Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency. J Allergy Clin Immunol, 2013. 131(6): p. 1604-10.
56. la Marca, G., et al., Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots. J Allergy Clin Immunol, 2014. 134(1): p. 155-9.
57. Ford, G. and S.H. LaFranchi, Screening for congenital hypothyroidism: A worldwide view of strategies. Best Practice & Research Clinical Endocrinology & Metabolism, 2014. 28(2): p. 175-187.
58. EURORDIS. Key Principles for Newborn Screening. 2021 [cited 2021 7 June]; Available from: https://www.eurordis.org/newbornscreening.
59. Markert, M.L., et al., Thymus transplantation in complete DiGeorge anomaly. Immunol Res, 2009. 44(1-3): p. 61-70.
60. Kreins, A.Y., et al., Correction of both immunodeficiency and hypoparathyroidism by thymus transplantation in complete DiGeorge syndrome. Am J Transplant, 2020. 20(5): p. 1447-1450.
61. Markert, M.L., et al., First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of 2 cases. Blood, 2011. 117(2): p. 688-96.
62. Collins, C., et al., Congenital Athymia: Genetic Etiologies, Clinical Manifestations, Diagnosis, and Treatment. J Clin Immunol, 2021. 41(5): p. 881-895.
63. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunological reviews, 2019. 287(1): p. 241-252.
64. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. J Allergy Clin Immunol, 2017. 139(3): p. 733-742.
65. Waisbren, S.E., et al., Effect of Expanded Newborn Screening for Biochemical Genetic Disorders on Child Outcomes and Parental Stress. JAMA, 2003. 290(19): p. 2564-2572.
66. Schmidt, J.L., et al., The impact of false-positive newborn screening results on families: a qualitative study. Genet Med, 2012. 14(1): p. 76-80.

SUPPLEMENTARY MATERIAL
SUPPLEMENTAL METHODS

Systematic review

A systematic review was conducted on NBS for SCID and (case) definitions used in pilot studies and population based screening. The findings were reported according to the principles outlined in Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (www. prisma-statement.org).

Literature search

An electronic search was performed on MEDLINE (PubMed), EMBASE (excluding MEDLINE), Cochrane library and Scopus databases on 16 February 2020. The keywords included synonyms for 'newborn screening' and 'primary immunodeficiencies/severe combined immunodeficiency/inborn errors of immunity'. Checking the reference lists of included studies identified additional publications. The search strategy is shown below in the Search Strategy.

Eligibility criteria

The initial search strategy was aimed at identification of all studies using definitions in NBS for SCID not discriminating between larger cohort studies and case studies. Systematic reviews, meta-analyses, randomized control trials, case reports, case-control studies, cohorts studies, letters to the editor without language restrictions were included in the initial search strategy. Exclusion criteria included publication of editorials, commentaries, unpublished manuscripts, dissertations, books and book chapters, lectures and speeches.

Study selection/Screening for inclusion

The study selection flow diagram is shown in Figure 1. Titles and abstracts of all the records retrieved from the electronic database searches were screened in a standardized manner. Records that were not clearly related to NBS for SCID and records that did not meet the eligibility criteria were excluded. Eligible articles were assed based on full text. Reviews were only included if classifications or definitions differed from previous original published articles. Case series describing retrospective TREC analysis of specific groups of PID patients were excluded.

Data extraction

Items that were extracted from included articles were: author, year, study design, study location, population based screening/pilot study, used TREC assay (in house or CE-IVD marked assays), using second tier test option after TREC analysis, TREC cut-off value, number of newborns screened, number of retest on initial NBS cards, number of repeat

DBS requested and number of referrals for follow-up diagnostics. If studies used an anonymized study population, presumptive positives rates were assumed referral rates. In addition, definitions on screening results, definitions or classifications used in screening algorithms and classifications of outcomes/diagnoses after follow-up were collected. Finally, definitions of premature birth and adjustments for premature infants in screening algorithms were extracted. The final selection of publications was discussed with the panel.

Quality assessment

The reviewer examined reports for completeness of reporting on screening outcomes, screening algorithms and classifications after follow-up. In some cases, multiple records described the same screening population. If the used definitions were consistent between these records, the most recent record was included. If information was missing in one of both studies, both articles would be included. Given the descriptive nature of this review, results were not combined for meta-analysis and a formal statistical analysis was not performed.

SEARCH STRATEGY

(conducted on February 16, 2021)

PubMed

#1 (28444 hits)

"Newborn screening"[tiab] OR "Neonatal screening"[tiab] OR ("Newborn"[tiab] AND "Screening"[tiab]) OR ("Neonatal"[tiab] AND "Screening"[tiab]) OR "Guthrie card"[tiab] OR "Guthrie test"[tiab] OR "Dried blood spot"[tiab] OR "Dried blood spots"[tiab] OR "T cell receptor excision circle"[tiab] OR "T cell receptor excision circles"[tiab] OR "T-cell receptor excision circle"[tiab] OR "T-cell receptor excision circles"[tiab] OR "TREC"[tiab] OR "TRECs"[tiab] OR "Kappa deleting recombination excision circle"[tiab] OR "Kappa deleting recombination excision circles"[tiab] OR "Kappa-deleting recombination excision circle"[tiab] OR "Kappa-deleting recombination excision circles"[tiab] OR "KREC"[tiab] OR "KRECs"[tiab] OR "Neonatal screening"[MeSH] OR "Dried Blood Spot Testing"[MeSH]

#2 (34356 hits)

"PID"[tiab] OR "primary immune deficiency"[tiab] OR "primary immune deficiencies"[tiab] OR "primary immunodeficiency"[tiab] OR "primary immunodeficiencies"[tiab] OR "SCID"[tiab] OR "Severe combined immunodeficiency"[tiab] OR "T cell lymphopenia"[tiab] OR "T cell lymphopenias"[tiab] OR "T-cell lymphopenia"[tiab] OR

"T-cell lymphopenias"[tiab] OR "T cell deficiencies"[tiab] OR "T-cell deficiency"[tiab] OR "T-cell deficiencies"[tiab] OR "T cell deficiency"[tiab] OR "inborn errors of immunity"[tiab] OR "Severe Combined Immunodeficiency"[MeSH] OR "Primary Immunodeficiency Diseases"[Mesh:NoExp]

#3 (515 hits)
#1 AND #2

EMBASE
#1 (10400 hits) AND [embase]/lim NOT ([embase]/lim AND [medline]/lim)
'Newborn screening':ti,ab OR 'Neonatal screening':ti,ab OR 'Guthrie card':ti,ab OR 'Guthrie test':ti,ab OR 'Dried blood spot':ti,ab OR 'Dried blood spots':ti,ab OR 'T cell receptor excision circle assay':ti,ab OR 'T cell receptor excision circles':ti,ab OR 'T-cell receptor excision circle assay':ti,ab OR 'T-cell receptor excision circles':ti,ab OR TREC:ti,ab OR TRECs:ti,ab OR 'Kappa deleting recombination excision circle':ti,ab OR 'Kappa deleting recombination excision circles':ti,ab OR 'Kappa-deleting recombination excision circle':ti,ab OR 'Kappa-deleting recombination excision circles':ti,ab OR #13 AND [embase]/lim NOT ([embase]/lim AND [medline]/lim)KREC:ti,ab OR KRECs:ti,ab OR 'Newborn screening'/exp OR 'Dried Blood Spot Testing'/exp

#2 (27614) AND [embase]/lim NOT ([embase]/lim AND [medline]/lim)
pid:ab,ti OR 'primary immune deficiency':ab,ti OR 'primary immune deficiencies':ab,ti OR 'primary immunodeficiency':ab,ti OR 'primary immunodeficiencies':ab,ti OR scid:ab,ti OR 'severe combined immunodeficiency':ab,ti OR 't cell lymphopenia':ab,ti OR 't cell lymphopenias':ab,ti OR 't-cell lymphopenia':ab,ti OR 't-cell lymphopenias':ab,ti OR 't cell deficiencies':ab,ti OR 't-cell deficiency':ab,ti OR 't-cell deficiencies':ab,ti OR 't cell deficiency':ab,ti OR 'inborn errors of immunity':ab,ti OR 'severe combined immunodeficiency'/exp OR 'primary immunodeficiency diseases'/mj

#3 (533 hits)
#1 AND #2

#4 (110 hits)
#3 AND ('article'/it OR 'article in press'/it OR 'conference review'/it OR 'editorial'/it OR 'erratum'/it OR 'letter'/it OR 'note'/it OR 'review'/it OR 'short survey'/it)

Cochrane

#1 (1112 hits)

"Newborn screening" or "Neonatal screening" or "Guthrie card" or "Guthrie test" or "Dried blood spot" or "Dried blood spots" or "T cell receptor excision circle" or "T cell receptor excision circles" or "T-cell receptor excision circle" or "T-cell receptor excision circles" or "TREC" or "TRECs" or "Kappa deleting recombination excision circle" or "Kappa deleting recombination excision circles" or "Kappa-deleting recombination excision circle" or "Kappa-deleting recombination excision circles" or "KREC" or "KRECs":ti,ab,kw

#2 (1688 hits)

"PID" or "primary immune deficiency" or "primary immune deficiencies" or "primary immunodeficiency" or "primary immunodeficiencies" or "SCID" or "Severe combined immunodeficiency" or "T cell lymphopenia" or "T cell lymphopenias" or "T-cell lymphopenia" or "T-cell lymphopenias" or "T-cell deficiency" or "T cell deficiency" or "T-cell deficiencies" or "T cell deficiencies":ti,ab,kw

#3 (8 hits)

#1 AND #2

SUPPLEMENTAL TABLES

Table S1. Study characteristics and details on included articles in the systematic review of definitions used in NBS for SCID

Table S2. Terminology and definitions of screening results used in studies on NBS for SCID

Table S3. Terminology and definitions of variables in the screening algorithm used in studies on NBS for SCID

Table S4. Classification of (case) definitions and outcomes after follow-up used in studies on NBS for SCID

Table S5. Terminology and definitions of preterm infants used in studies on NBS for SCID

Available Online via: https://www.jacionline.org/cms/10.1016/j.jaci.2021.08.026/attachment/3b7278a7-ad9c-4fa5-bceb-962d4cda2043/mmc1.pdf

SUPPLEMENTAL REFERENCES

1. Thorsen J, Kolbert K, Joshi A, Baker M, Seroogy CM. Newborn Screening for Severe Combined Immunodeficiency: 10-Year Experience at a Single Referral Center (2009-2018). J Clin Immunol. 2021.
2. Richards S, Gennery AR, Davies EG, Wong M, Shaw PJ, Peake J, et al. Diagnosis and management of severe combined immunodeficiency in Australia and New Zealand. Journal of Paediatrics and Child Health. 2020;56(10):1508-13.
3. Giżewska M, Durda K, Winter T, Ostrowska I, Ołtarzewski M, Klein J, et al. Newborn Screening for SCID and Other Severe Primary Immunodeficiency in the Polish-German Transborder Area: Experience From the First 14 Months of Collaboration. Front Immunol. 2020;11:1948.
4. Blom M, Bredius RGM, Jansen ME, Weijman G, Kemper EA, Vermont CL, et al. Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J Clin Immunol. 2021;41(1):99-108.
5. Strand J, Gul KA, Erichsen HC, Lundman E, Berge MC, Trømborg AK, et al. Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol. 2020;11:1417.
6. Argudo-Ramírez A, Martín-Nalda A, Marín-Soria JL, López-Galera RM, Pajares-García S, González de Aledo-Castillo JM, et al. First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain). Front Immunol. 2019;10:2406.
7. Thomas C, Durand-Zaleski I, Frenkiel J, Mirallié S, Léger A, Cheillan D, et al. Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. Clinical Immunology. 2019;202:33-9.
8. Audrain MAP, Léger AJC, Hémont CAF, Mirallié SM, Cheillan D, Rimbert MGM, et al. Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). Journal of Clinical Immunology. 2018;38(7):778-86.
9. Amatuni GS, Currier RJ, Church JA, Bishop T, Grimbacher E, Nguyen AA, et al. Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics. 2019;143(2).
10. Kobrynski LJ. Identification of non–severe combined immune deficiency T-cell lymphopenia at newborn screening for severe combined immune deficiency. Annals of Allergy, Asthma & Immunology. 2019;123(5):424-7.
11. Nourizadeh M, Shakerian L, Borte S, Fazlollahi M, Badalzadeh M, Houshmand M, et al. Newborn screening using TREC/KREC assay for severe T and B cell lymphopenia in Iran. Scand J Immunol. 2018:e12699.
12. Al-Mousa H, Al-Dakheel G, Jabr A, Elbadaoui F, Abouelhoda M, Baig M, et al. High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. Front Immunol. 2018;9:782.
13. Routes J, Verbsky J. Newborn Screening for Severe Combined Immunodeficiency. Current Allergy and Asthma Reports. 2018;18(6):34.

14. Rechavi E, Lev A, Simon AJ, Stauber T, Daas S, Saraf-Levy T, et al. First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol. 2017;8:1448.
15. Rechavi E, Lev A, Saraf-Levy T, Etzioni A, Almashanu S, Somech R. Newborn Screening for Severe Combined Immunodeficiency in Israel. International Journal of Neonatal Screening. 2017;3(2):13.
16. Son S, Kang J-M, Kim JM, Sung S, Kim Y-S, Lee H, et al. The First Newborn Screening Study of T-Cell Receptor Excision Circle and κ-Deleting Recombination Excision Circle for Severe Combined Immunodeficiency in Korea: A Pilot Study. Pediatr Infect Vaccine. 2017;24(3):134-40.
17. Kanegae MPP, Barreiros LA, Sousa JL, Brito MAS, Oliveira EBJ, Soares LP, et al. Newborn Screening for Severe Combined Immunodeficiencies using TRECs and KRECs: Second Pilot Study in Brazil. Rev Paul Pediatr. 2017;35(1):25-32.
18. Kanegae MPP, Barreiros LA, Mazzucchelli JTL, Hadachi SM, de Figueiredo Ferreira Guilhoto LM, Acquesta AL, et al. Neonatal screening for severe combined immunodeficiency in Brazil. Jornal de Pediatria. 2016;92(4):374-80.
19. Tagliaferri L, Kunz JB, Happich M, Esposito S, Bruckner T, Hübschmann D, et al. Newborn screening for severe combined immunodeficiency using a novel and simplified method to measure T-cell excision circles (TREC). Clinical Immunology. 2017;175:51-5.
20. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, et al. Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol. 2017;37(1):51-60.
21. Zetterström RH, Barbaro M, Ohlsson A, Borte S, Jonsson S, Winiarski J, et al. Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden. International Journal of Neonatal Screening. 2017;3(2):11.
22. De Felipe B, Olbrich P, Goycochea-Valdivia W, Delgado-Pecellin C, Sanchez-Moreno P, Sánchez B, et al. Newborn Screening for Primary T- and B-Cell Immune Deficiencies—A Prospective Study in Andalucía. International Journal of Neonatal Screening. 2017;3(4):27.
23. de Felipe B, Olbrich P, Lucenas JM, Delgado-Pecellin C, Pavon-Delgado A, Marquez J, et al. Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol. 2016;27(1):70-7.
24. Blom M, Pico-Knijnenburg I, Sijne-van Veen M, Boelen A, Bredius RGM, van der Burg M, et al. An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. Clin Immunol. 2017;180:106-10.
25. Chien Y-H, Yu H-H, Lee N-C, Ho H-C, Kao S-M, Lu M-Y, et al. Newborn Screening for Severe Combined Immunodeficiency in Taiwan. International Journal of Neonatal Screening. 2017;3(3):16.
26. Madkaikar M, Aluri J, Gupta S. Guidelines for Screening, Early Diagnosis and Management of Severe Combined Immunodeficiency (SCID) in India. The Indian Journal of Pediatrics. 2016;83(5):455-62.
27. Chien YH, Chiang SC, Chang KL, Yu HH, Lee WI, Tsai LP, et al. Incidence of severe combined immunodeficiency through newborn screening in a Chinese population. J Formos Med Assoc. 2015;114(1):12-6.
28. Kwan A, Hu D, Song M, Gomes H, Brown DR, Bourque T, et al. Successful newborn screening for SCID in the Navajo Nation. Clin Immunol. 2015;158(1):29-34.

29. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. Jama. 2014;312(7):729-38.
30. Audrain M, Thomas C, Mirallie S, Bourgeois N, Sebille V, Rabetrano H, et al. Evaluation of the T-cell receptor excision circle assay performances for severe combined immunodeficiency neonatal screening on Guthrie cards in a French single centre study. Clin Immunol. 2014;150(2):137-9.
31. Adams SP, Rashid S, Premachandra T, Harvey K, Ifederu A, Wilson MC, et al. Screening of neonatal UK dried blood spots using a duplex TREC screening assay. J Clin Immunol. 2014;34(3):323-30.
32. Vogel BH, Bonagura V, Weinberg GA, Ballow M, Isabelle J, DiAntonio L, et al. Newborn screening for SCID in New York State: experience from the first two years. J Clin Immunol. 2014;34(3):289-303.
33. Kwan A, Church JA, Cowan MJ, Agarwal R, Kapoor N, Kohn DB, et al. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia in California: results of the first 2 years. J Allergy Clin Immunol. 2013;132(1):140-50.
34. Borte S, von Döbeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood. 2012;119(11):2552-5.
35. Verbsky JW, Baker MW, Grossman WJ, Hintermeyer M, Dasu T, Bonacci B, et al. Newborn screening for severe combined immunodeficiency; the Wisconsin experience (2008-2011). J Clin Immunol. 2012;32(1):82-8.
36. Comeau AM, Hale JE, Pai S-Y, Bonilla FA, Notarangelo LD, Pasternack MS, et al. Guidelines for implementation of population-based newborn screening for severe combined immunodeficiency. Journal of Inherited Metabolic Disease. 2010;33(S2):273-81.
37. Baker MW, Laessig RH, Katcher ML, Routes JM, Grossman WJ, Verbsky J, et al. Implementing routine testing for severe combined immunodeficiency within Wisconsin's newborn screening program. Public Health Rep. 2010;125 Suppl 2(Suppl 2):88-95.
38. Routes JM, Grossman WJ, Verbsky J, Laessig RH, Hoffman GL, Brokopp CD, et al. Statewide newborn screening for severe T-cell lymphopenia. Jama. 2009;302(22):2465-70.
39. Shearer WT, Dunn E, Notarangelo LD, Dvorak CC, Puck JM, Logan BR, et al. Establishing diagnostic criteria for severe combined immunodeficiency disease (SCID), leaky SCID, and Omenn syndrome: The Primary Immune Deficiency Treatment Consortium experience. Journal of Allergy and Clinical Immunology. 2014;133(4):1092-8.

CHAPTER 11

Future perspectives of newborn screening for inborn errors of immunity

Review

Maartje Blom, Robbert G,M. Bredius and Mirjam van der Burg

International Journal of Neonatal Screening, 2021; 7(4): 74

ABSTRACT

Newborn screening (NBS) programs continue to expand due to innovations in both test methods and treatment options. Since the introduction of the T-cell receptor excision circle (TREC) assay 15 years ago, many countries have adopted screening for severe combined immunodeficiency (SCID) in their NBS program. SCID became the first inborn error of immunity (IEI) in population-based screening and at the same time the TREC assay became the first high-throughput DNA-based test in NBS laboratories. In addition to SCID, there are many other IEI that could benefit from early diagnosis and intervention by preventing severe infections, immune dysregulation and autoimmunity, if a suitable NBS test was available. Advances in technologies such as KREC analysis, epigenetic immune cell counting, protein profiling and genomic techniques such as next-generation sequencing (NGS) and whole-genome-sequencing (WGS) could allow early detection of various IEI shortly after birth. In the next years, the role of these technical advances as well as ethical, social, and legal implications, logistics and cost will have to be carefully examined before different IEI can be considered as suitable candidates for inclusion in NBS programs.

INTRODUCTION

Expansion of newborn screening (NBS) with new disorders is driven by development of new test modalities and treatment options. One of the more recent developments was the introduction of the first high-throughput DNA-based NBS test in the screening laboratory for the detection of severe combined immunodeficiency (SCID). SCID is one of the most severe forms of inborn errors of immunity (IEI) characterized by the absence or dysfunction of T-lymphocytes affecting both cellular and humoral immunity [1, 2]. SCID or Combined immunodeficiency (CID), which is generally less profound than SCID, is a term used to describe a variety of genetic defects in more than 50 genes [3, 4]. Infants with SCID typically appear normal at birth but develop severe infections in the first months of life. Without curative treatment, in the form of allogeneic hematopoietic stem cell transplantation (HSCT) or in some specific forms of SCID, gene therapy, affected infants die within the first year of life. Clearly, early definitive treatment, before the onset of infections, has the best outcome [5, 6]. Due to the severity of the disease, an asymptomatic status early in life and improved survival and outcome after an early diagnosis, SCID was considered a suitable candidate for NBS. NBS for SCID is based on the detection of T-cell receptor excision circles (TRECs) with (q)PCR. TRECs are formed as a byproduct in approximately 70% of developing αβ T-lymphocytes and can therefore serve as a marker for thymic output [7, 8]. Since the introduction of the TREC assay 15 years ago, many countries have introduced SCID in their NBS programs leading to improved outcomes for SCID patients worldwide [6, 9].

SCID became the first immune disorder in the NBS program. However, in addition to SCID, there are many other IEI that could benefit from early diagnosis and intervention if a suitable NBS test was available. IEI are an heterogenous group of disorders characterized by an increased susceptibility to severe and/or recurrent infections, due to genetic defects affecting development and/or function of the immune system [10]. Autoimmunity, autoinflammation, allergy, and malignancy can be common, and in some cases, predominant, clinical manifestations [11]. More than 430 IEI have been described with the discovery of new IEI occurring at an impressive rate [12]. With the Wilson and Jungner screening criteria in mind, several IEI would qualify as serious conditions that cause an important health problem and would benefit from early detection and treatment by preventing severe infections, immune dysregulation and auto-immunity [13, 14]. For some monogenetic IEI allogeneic HSCT might be a curative approach and autologous gene therapy could serve as a possible alternative treatment in the future [15]. This review will present future perspectives and recent technological advances that can potentially lead to expanded NBS for IEI.

Newborn screening for (X-linked) agammaglobulinemia

One of the first steps in the expansion of NBS programs for IEI would be the implementation of NBS for X-linked agammaglobulinemia (XLA) and autosomal recessive XLA-like disorders. Agammaglobulinemia refers to a group of IEI in which B-cells are absent or dysfunctional, resulting in severely decreased or absent levels of all classes of serum immunoglobulins (Igs) and an inability to produce specific antibodies [16]. The most common form of this disease is XLA, caused by mutations in the Bruton's tyrosine kinase (BTK) gene. BTK is a signal-transducing protein, thus mutations in the BTK gene cause a block in the differentiation of B-cell progenitors into mature B-cells affecting humoral immunity [17-19]. Patients with agammaglobulinemia develop serious recurrent infections from sixth months of life, predominantly in the respiratory tract and the gastrointestinal tract [20, 21]. Moreover, patients are at risk for severe meningoencephalitis caused by enteroviruses or life-threatening sepsis [22-24]. Without treatment, agammaglobulinemia can lead to chronic lung disease and permanent lung damage, such as bronchiectasis, and even premature mortality due to severe infections and complications [22, 25, 26]. Treatment consists of life-long administration of Igs either intravenously or subcutaneously combined with prophylactic antibiotics if indicated [27]. Early detection of these severe B-cell deficiencies and timely initiation of Ig replacement therapy is crucial to prevent secondary complications, long-term morbidity and consequently mortality [25, 28]. Previous studies have shown an increased incidence of chronic lung disease in patients with delayed diagnosis with a significant impact on prognosis and quality of life suggesting that NBS for XLA and other B-cell deficiencies would almost certainly result in improved clinical outcomes and health gain [26]. However, studies are lacking that demonstrate conclusive evidence that an early diagnosis is associated with decreased morbidity and mortality rates in a large cohort of agammaglobulinemia patients. Severe B-cell deficiencies can be detected by measuring kappa-deleting recombination excision circles (KRECs) in dried blood spots (DBS). Similar to T-cells, B-cells undergo V(D)J recombination to develop unique B-cell antigen receptors which also yields an excision circle: the KREC, serving as an indirect marker for the presence of B-cells [7, 29]. KRECs can be measured simultaneously with TRECs in a multiplex qPCR-based assay allowing a swift implementation of KREC detection in the NBS laboratory at relatively low cost [30]. Detection of XLA and other B-cell deficiencies by KREC quantification in DBS has already been proven to be successful by several NBS pilot studies [31-35]. The reason why countries are not moving forward with NBS for B-cell deficiencies while a suitable test is available, is probably due to the relatively high referral rate associated with KREC screening (due to prematurity, maternal immunosuppressant use etc.) and the lack of conclusive evidence of substantive health gain by early diagnosis of agammaglobulinemia. NBS for SCID The Dutch Health Council proposed XLA as a

potentially suitable candidate for NBS in 2015, but considered detailed identification of the exact characteristics of the test in routine neonatal screening a requirement [36]. Even though the referral rate in KREC screening would be depending on the chosen cut-off value, there is need to evaluate second tier options including epigenetic immune cell counting and next generation sequencing (NGS) after KREC analysis. Finally, a retrospective multi-center study comparing clinical outcomes and quality of life of patients with an early and late diagnosis of XLA and other B-cell deficiencies will help NBS programs to move forward towards universal NBS for agammaglobulinemia resulting in health gain for these patients worldwide.

Epigenetic immune cell counting: a new player in the field

Not all IEI and immune dysregulation disorders can be detected by absent TRECs or KRECs. With epigenetic immune cell counting, quantitative defects of immune cell populations such as T-cells, B-cells, regulatory T-cells (Tregs) and neutrophils could offer early detection of several IEI shortly after birth [37] . Epigenetic immune cell counting is a technique based on amplification of cell-specific demethylated genomic regions with qPCR allowing measurement of relative cell counts in DBS as depicted in Figure 1 [37].

Absence of TRECs is a highly sensitive marker for SCID and epigenetic immune cell counting could only match this sensitivity if naïve T-cells or recent thymic emigrants (RTEs) could be quantified by epigenetic qPCR, i.e., to detect SCID cases with maternal engraftment [2, 38]. Combined immunodeficiencies such as ZAP-70 deficiency or Major Histocompatibility Complex (MHC) class I and II gene expression deficiency cannot be detected with the TREC assay as T-cell development is intact beyond the point of T-cell receptor (TCR) gene recombination [34]. MHC class I deficiency is characterized by a decreased surface expression of HLA class I molecules leading to decreased numbers of circulating CD8+ αβ T-cells, chronic infections in the respiratory tract and skin granulomatous lesions. Prevention and treatment of bronchial infections are the main therapeutic strategies for these patients [39]. MHC class II deficiency leads to an impaired antigen presentation by antigen presenting cells and an incomplete maturation of CD4+ T-cells. Early diagnosis of MHC class II deficiency is important to enable HSCT before irreversible organ damage secondary to recurrent infections has occurred [40]. As these CIDs usually present with low CD4+ or CD8+ T-cells, some patients could be identified with epigenetic immune cell counting shortly after birth. With epigenetic immune cell counting, NBS for XLA and other B-cell deficiencies based on quantification of relative B-cell counts could have a higher positive predictive value in comparison to KREC detection in DBS. Relative B-cell counts are after all a more direct marker for absolute B-cell counts than KRECs. By determining relative numbers

of FOXP3+ Tregs, immune dysregulation disorders characterized by low Tregs could be identified in the neonatal phase. Monogenic autoimmune disorders caused by inborn errors in Tregs can have variable clinical manifestations, ranging from early-onset severe autoimmunity to late-onset or atypical symptoms [41]. Patients with an early-onset, severe phenotype require immediate therapy including immunosuppression followed by HSCT [42]. However, Treg numbers and function can be impaired by various underlying causes and NBS based on detection of FOXP3+ Tregs might be of limited value. In addition, quantification of relative Treg cell counts might not be an option for patients who express fairly normal amounts of mutated FOXP3 protein which is the case in some Immune dysregulation- Polyendocrinopathy- Enteropathy- X-linked (IPEX) syndrome patients [43]. Epigenetic immune cell counting did reveal increased levels of demethylation in the *FoxP3* gene locus in symptomatic IPEX patients, potentially serving as a diagnostic aid [37]. With the relative quantification of neutrophils in DBS, severe congenital neutropenia (SCN) and other conditions associated with severe neutropenia at birth could be identified via NBS. Patients with SCN are characterized by impaired maturation of neutrophil granulocytes leading to recurrent, life-threatening infections and predisposition to myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML) [44]. Daily subcutaneous G-CSF administration will lead to a reduction of infections, drastically improving quality of life. HSCT can serve as a curative treatment option for SCN patients who are nonresponsive to G-CSF therapy, patients who develop MDS or AML and patients with mutations in genes predisposing for malignant transformation (e.g. *CSF3R* or *RUNX1*) [45, 46]. Prevention of infections would be the main purpose of early identification of SCN patients malignant transformation would occur at later stage. A major challenge to overcome in NBS for SCN would be the high number of secondary findings as neutropenia is frequently observed in neonates with maternal pre-eclampsia, sepsis, twin-twin transfusion, alloimmunization, and hemolytic disease being the most common causes [47]. Second tier testing with NGS might be an option to overcome this exceeding number of referrals [48]. In addition to NGS, repeating neutrophil measurements with epigenetic immune cell counting in second heel prick cards after one to two weeks could also reduce the high number of referrals as many of the above-mentioned causes of neonatal neutropenia will resolve shortly after birth. Figure 2 shows some additional immune types that can be identified with epigenetic immune cell counting and their corresponding quantitative defects or IEI. In addition to population based screening, retrospectively applying epigenetic immune cell counting to NBS cards could allow the identification of neonatal prognostic markers for a range of disorders. The technique also facilitates diagnostics or monitoring in resource-poor regions, where logistics for appropriate cell counting is hampered as blood collection and measurement cannot be performed in close succession [37]. A pitfall of measuring relative cell counts in contrast to absolute cell counts as measured

via flow cytometric immunophenotyping, is that proportional cell numbers within the corresponding reference range might not accurately reflect the clinically relevant alterations in the patient. Patients could have very low numbers of total leukocytes with normal percentage of T-cells concealing a severe T-cell lymphopenia. Before epigenetic immune cell counting could be applied as a first tier test in the screening laboratory, automating the protocol would be required to increase the throughput time and to enable analysis of more samples with less hands-on-time.

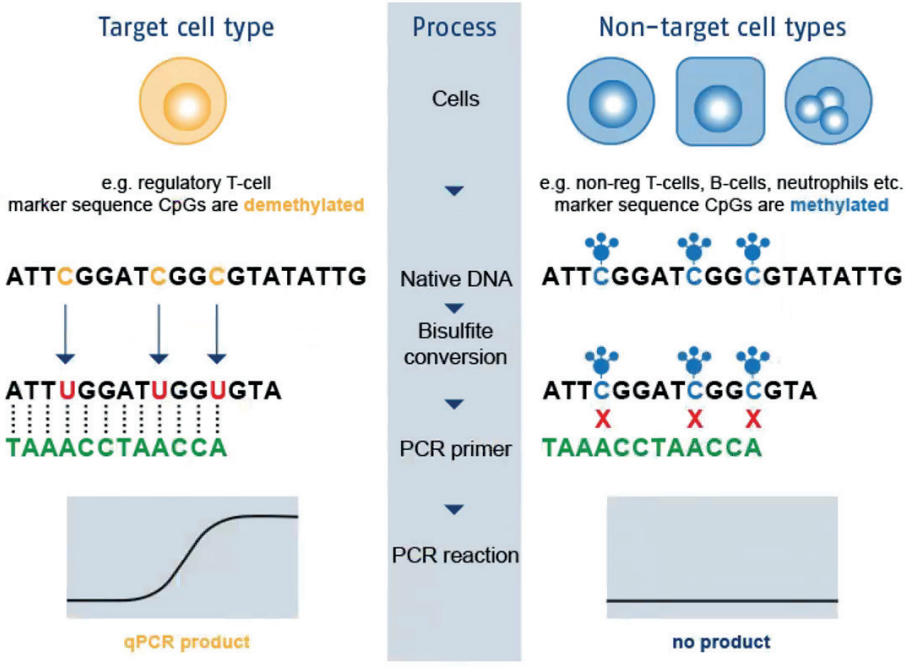

Figure 1. Epigenetic immune cell counting. Unique cell type–specific DNA methylation markers were identified. After bisulfite conversion of the genomic DNA, unmethylated CpG dinucleotides are converted and amplified to TpGs, whereas methylated CpGs remain unaltered. Bisulfite conversion translates epigenetic markers into sequence information, allowing immune cell quantification with qPCR [37]. Figure from Epimune GmbH, Berlin, Germany.

Newborn screening for interferonopathies

Another group of IEI potentially suitable for future NBS are type I interferonopathies. Type I interferonopathies encompass a spectrum of rare, genetic disorders that are characterized by autoinflammation and chronic type I interferon (IFN) production in the absence of a viral infection. In addition to elevated type I IFN levels, these disorders are characterized by calcifications in the central nervous system, leukoencephalopathy,

severe developmental delay, and skin lesions. Because of the severity of these diseases, patients usually do not survive into adulthood [49, 50]. Elevated type I IFN levels lead to an increase in 'IFN-stimulated genes' (ISGs). These ISGs can easily be monitored through quantitative PCR in peripheral blood. The results of a panel of six ISGs can be combined into an IFN-score and this assay has been proposed as the 'gold standard' for the diagnosis of pediatric patients suffering from interferonopathies [51]. A recent multi-national study successfully showed that the IFN-score can be measured in DBS of newborns, allowing detection of type I interferonopathies shortly after birth [52]. Case reports with experimental treatments such as nucleoside reverse transcriptase inhibitors (NRTIs), IFN and IFN receptor blocking antibodies, and JAK1 inhibitors have suggested that early treatment may inhibit or delay developmental decline and disease progression [53]. More evidence will need to be collected on the effectiveness of these experimental treatments as actionability is one of the major criteria when considering conditions for NBS programs. In addition, the specificity of the IFN-score as a NBS test should be determined in the context of patients with a viral infection who can also present with an evident IFN-score [50].

Protein-based newborn screening for IEI

In addition to DNA-based techniques, protein-based methods can also serve as potentially suitable screening tests for some IEI. Protein profiling has been described as a technique to broaden NBS for IEI with screening for innate immunity defects [54, 55]. Recently, a NBS test based on suspension bead arrays for protein profiling has been to described to detect 22 disorders due to defects in the complement system or phagocytic function prior to the onset of clinical symptoms [56]. Accurate and early diagnosis of these patients is important as complement deficiencies and phagocytic disorders are associated with numerous immunological complications. Complement deficiencies give rise to a variable clinical phenotype including recurrent and persistent infections, hereditary angioedema and autoimmune complications. Disorders of granulocyte number and function lead to delayed wound healing, severe infections, abscess formation and inflammatory manifestations (e.g. colitis in Chronic Granulomatous Disease) [3]. Early diagnosis of such disorders allows immediate clinical intervention and prevention of severe morbidity and mortality. Some phagocytic diseases might even qualify for HSCT or in the future, gene therapy. A proteomic screening approach using tandem mass spectrometry was additionally described to quantify signature peptides for BTK, WASP, and T-cell marker CD3ε to screen for XLA, Wiskott-Aldrich Syndrome (WAS), and SCID, respectively [57]. WAS is a rare, X-linked IEI characterized by recurrent infections, microthrombocytopenia, eczema, and an increased incidence of autoimmunity and malignancies [58]. Mutations in the *WAS* gene have various effects on the level of WASp correlating to the severity of the

disease. The absence of functional WASp can lead to fatal outcomes if not diagnosed and treated early in life with HSCT [58]. The selected reaction monitoring (immuno-SRM) technology further enhanced the sensitivity of quantifying IEI specific peptides with tandem mass spectrometry [59]. Recently, the proteomic panel was expanded to eight signature peptide biomarkers to screen for five molecularly defined IEI including adenosine deaminase (ADA) deficiency, Dedicator of cytokinesis 8 (DOCK8) deficiency, X-Linked Chronic Granulomatous disease (XL-CGD), WAS and XLA [60]. These IEI are strong candidates for inclusion in NBS programs as these disorders have effective treatment options, are well studied with good understanding of the clinical course and immuno-SRM is highly suitable as a high-throughput test in the NBS laboratory. A key benefit of protein-profiling is the notable number of IEI-associated proteins that can be examined in parallel using a limited amount of sample material.

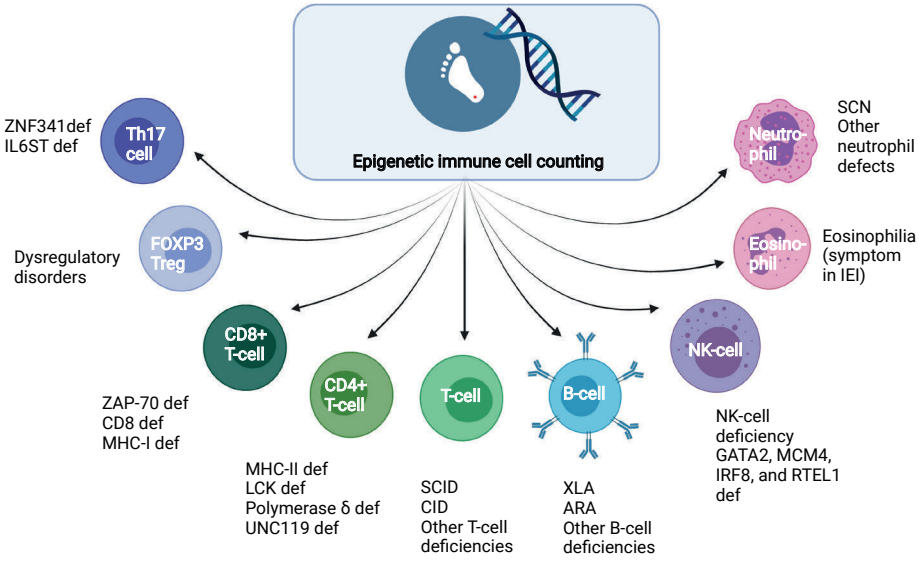

Figure 2. Different types of immune cells that can be identified with epigenetic immune cell counting and examples of corresponding quantitative defects or IEI. *Th17 – T helper 17, def - deficiency, IL6ST - IL6 signal transducer, ZNF341 - Zinc Finger Protein 341, FOXP3 - forkhead box P3, Treg – regulatory T-cell, ZAP-70 - Zeta-chain-associated protein kinase 70, MHC - major histocompatibility complex, SCID- severe combined immunodeficiency, CID – combined immunodeficiency, XLA – X-linked agammaglobulinemia, ARA- autosomal recessive agammaglobulinemia, NK-cell – natural killer cell, MCM4 - minichromosome maintenance complex component 4, IRF8 -interferon regulatory factor 8, RTEL1 - regulator of telomere elongation helicase 1, SCN – severe congenital neutropenia, IEI – inborn error of immunity.*

Genomic-based newborn screening for IEI

Even though TREC screening was the first high-throughput DNA technique in the screening laboratory, targeted DNA sequencing is already used as a tiered screening strategy for cystic fibrosis [61, 62]. Targeted DNA sequencing has also been described as a potential method to identify infants with familial hemophagocytic lymphohistiocytosis (FHLH) due to homozygous *UNC13D* inversion mutations [63]. Patients with HLH present with life-threatening inflammatory responses secondary to impaired lymphocyte functions. Clinical manifestations can include fever, splenomegaly, cytopenia, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis, low or absent NK-cell activity, hyperferritinemia and elevated levels of soluble IL-2 receptor [64]. Early diagnosis is crucial to prevent severe disease manifestations by timely initiation of first-line treatment, to determine the need for HSCT and to reduce possible post-HSCT sequelae [65]. There are several genes associated with FHLH [3], therefore in order to identify all variants in genes associated with FHLH, other DNA-based techniques such as NGS should be considered.

Recent technological advances in genomic medicine have led to the availability of rapid and inexpensive genomic sequencing techniques, including NGS, whole-exome sequencing (WES) and whole-genome sequencing (WGS). TREC/KREC screening is unable to detect many serious IEI and immune dysregulatory disorders and sequencing could provide a potential method for screening a wider array of health conditions. The increased use of NGS, WES and WGS in diagnostics raises the question whether these sequencing techniques could be applied in a screening context. Genomic-based NBS may be especially applicable to the detection of IEI, as these represent a heterogeneous group of conditions with varying clinical phenotypes. In addition, many IEI are monogenic, some of which may be difficult to diagnose clinically, and most can benefit from early medical interventions [66, 67]. Given the genetic and phenotypic heterogeneity of IEI, screening all of these diseases would require a range of different test modalities, which is unfeasible from a logistic or economic perspective in the context of NBS. Applying genomic sequencing techniques in NBS would allow parallel testing, using one platform to detect many clinically actionable diseases [14]. The future role of genomic technology in NBS for IEI has previously been discussed extensively, therefore this review will summarize the discussion points [14, 68, 69].

There are programs who have already successfully adopted NGS in their screening programs for SCID, primarily as a second tier test after TREC analysis [70, 71]. NGS with targeted gene panels on DBS will facilitate and accelerate final molecular diagnoses of affected newborns while providing useful information for management and follow-up. Previously, the time from sample collection to NGS results took weeks to months,

but targeted NGS has a rapid turn-around time (results within 2 – 3 working days) [70]. Additionally, a higher TREC cut-off value in combination with NGS allows the detection of atypical and leaky SCID with potentially higher TREC values, but a clear HSCT indication based on immunophenotyping. On the other hand, NGS is associated with relatively high analyses and equipment costs and a cost-effectiveness analysis including efficiency gains and improved management could help NBS policy makers when discussing implementation of NGS [72]. The successful implementation of NGS in NBS as a second tier has opened the discussion for expansion of NBS for IEI by using sequencing techniques as a first tier [69]. NGS is very adaptable and could serve as a first tier test to screen for monogenetic disease, however exome based targeted NGS will not be able to identify IEI with variants in genes not included in the gene panels or IEI with structural variants or intronic variants. Experts prefer WGS approaches as they are able to simultaneously sequence both intronic and exonic regions [14]. Although a proof-of concept study for a WGS-based approach in screening for IEI has already been published, WGS poses significant challenges in the context of NBS [67]. Major concerns include the interpretation and management of large amounts of genetic data and ethical implications of incidental findings and carrier status for patients and other family members. In addition, genome-scale sequencing would require modification of current informed consent procedures [69, 73]. Pathogenicity interpretation and assessing the potential deleterious effects of novel variants remains challenging. Even with automated methodology allowing high-throughput analysis of large amounts of genomic data, manual review would still be required to define benign and pathogenic variants [67]. This is a labor-intensive, costly process and this type of expertise is currently not present in the majority of NBS laboratories. In addition, there is need to improve accuracy and completeness of reference databases and new methods for pathogenicity predication are necessary before genomic testing can be incorporated into NBS programs.

Policy makers, NBS practitioners, clinicians, and parents have also raised social concerns about expansion of NBS with WGS regarding privacy, trust, and desire for control over one's own and one's child's genomic information [74]. Parents seem to have an overall optimistic and enthusiastic orientation towards genomic advances in NBS, but they expressed concerns about privacy and control over test results [74-76]. Genetic profiling and potential genetic discrimination are important aspects that would need to be addressed [77]. Due to limited trust in the medical system and the NBS programs, parents would desire more clarity over the data produced with genomic technologies. At this point, NBS stakeholders are uncertain how to manage unintended findings unrelated to actionable disorders and how to establish criteria for the evaluation and incorporation of new disorders. NBS programs and pediatricians will

be responsible for follow-up of a greater number of conditions, as well as implementing an informed-consent process and management of the genomic data produced by the test [74]. All technical challenges as well as ethical, policy and clinical practice issues must be taken into consideration before adapting genomic technologies in population-based screening programs.

The adoption of TREC analysis and qPCR technology by NBS laboratories will enable further expansion of genomic techniques in NBS laboratories. However, several limitations, challenges and important considerations must be addressed prior to routine implementation of genomic technologies in NBS programs. Many of the questions posed above remain unanswered and must be further evaluated and clarified in prospective studies assessing the entire screening process including ethical, legal and social implications [69]. Screening without including any phenotypical markers as a first tier option remains challenging due to the rarity of IEI, missing links between gene defects and disease mechanisms and the inability to distinguish underlying pathogenic variants from the high number of genomic variations [52]. With genome-wide association studies relations between phenotype traits and genotype in IEI might be unraveled. In my opinion, in the near future, genomic technology will not be used as a primary 'standalone' screening approach, but as an addition to current screening methodologies.

Future newborn screening for SCID

In the near future, SCID will be implemented by an increasing number of NBS programs worldwide. However, as the TREC assay is a relatively expensive technique, implementation of this method in screening laboratories might be challenging for countries with less resources. NBS for SCID is particularly important in some of these countries; for example in Middle Eastern countries where the incidence of IEI is expected to be 20 times higher than in North America or Europe due to the relatively high incidence of consanguinity [78]. Development of new, low-cost technologies for testing newborns for a broad range of conditions is key in this process, while commercial initiatives for innovative pricing of reagents and equipment can be of aid as well. Experts from various disciplines should contribute their time for training and sharing expertise on an international level [79]. The importance of screening programs cannot be outweighed, however due to the lack of resources, educational programs and public awareness campaigns might be a more feasible option in the direct future. In the absence of NBS, clinicians should be aware of the early manifestations of SCID to enable an early diagnosis and timely intervention [80]. Close partnership of NBS programs, policy makers, immunologists, and HSCT specialists and sharing of experiences internationally could help to improve outcomes for SCID patients on a global level.

While some NBS program are still awaiting governmental decisions with regard to SCID screening, NBS programs that have already implemented SCID should continue to improve their current practice. With TREC screening, new cut-off values, adjusted screening algorithms and inclusion of second tier tests should be considered to increase positive predictive value and to reduce the number of false-positive referrals [81]. In addition, follow-up after an abnormal screening result will need to be further optimized as previous studies have shown that a relatively large part of SCID patients identified with NBS still developed infections prior to HSCT [82]. Time required to obtain TREC results, to refer a newborn to a pediatric-immunologist and to obtain results of confirmatory testing should be reduced to prevent significant delay in initiating protective measures. Best practice for isolation and antimicrobial prophylaxis to minimize infection exposure pre-HSCT should be harmonized across centers [83].

In the coming years, more NBS programs might shift from SCID as the primary target towards screening for actionable T-cell lymphopenia with low TRECs. NBS with TREC testing correlates with having recently formed T-cells in peripheral blood; therefore, one could argue that in TREC-based screening primary targets should include all serious, actionable T-cell deficiencies that are associated with low TRECs at birth. Parents believe that the term *actionable* includes conditions (1) where early interventions lead to health gain for the newborn, (2) where early diagnosis avoids the lengthy diagnostic odyssey and (3) where parents will have reproductive options during subsequent pregnancies [84]. For many health care providers, the definition of actionability in NBS is more limited to the management of the individual affected with the condition. The term actionable indicates that an urgent (early) intervention is required by a specialist and that the intervention results in a demonstrable improvement in outcome. Cases of significant T-cell lymphopenia that might benefit from antibiotic prophylaxis and protective isolation should be deemed actionable. The term actionable is more suitable than the term treatable, as withholding live-attenuated vaccines is an important early intervention leading to improved outcomes, given that vaccine-strain organisms such as BCG can cause serious infections in individuals with T-cell defects [85, 86].

In the next five years, many NBS programs will have implemented a multiplex PCR, measuring TRECs simultaneously with KRECs and *SMN1* introducing NBS for XLA and spinal muscular atrophy (SMA) [87]. The addition of KRECs will also be of value to SCID screening as it may assist in distinguishing B-/B+ phenotypes in SCID patients, therefore aiding in the diagnostic process. Some leaky or delayed-onset SCID patients, in particular T-B- SCID patients with hypomorphic mutations in DNA repair or cellular metabolism, might not be detected with TREC quantification [88]. The increase of toxic metabolites can well be tolerated to a certain degree by dividing T-cells, whereas

B-cells seem to be more vulnerable for genomic stress, for example in patients with delayed-onset ADA-SCID. For these patients, SCID screening will be further extended to tandem mass spectrometry measuring adenosine and deoxyadenosine for ADA deficient patients or purine nucleosides and 2'-deoxy-nucleosides for PNP deficient patients. Previous studies have shown that screening with tandem mass spectrometry was able to identify these infants at low cost [89-92].

After these developments, epigenetic immune cell counting will hopefully be optimized as a high-throughput test for the NBS program enabling NBS for a range of IEI. We might be a long way from first-tier WGS-based screening in newborns, but more and more countries will include NGS in their NBS programs as a second tier test in the next decade. Decades from now, NBS for IEI will enter the genomic era. Genome wide associations studies may have identified an exceeding number of associations between variants and phenotypes, explaining the contribution of common variants to variable penetrance and phenotypic complexity in IEI [93]. Reference databases will be more complete and pathogenicity prediction programs will demonstrate improved accuracy. The future of NBS holds many uncertainties, but one thing is sure, with all these technological advances, exciting times are waiting for population-based screening programs.

CONCLUSIONS

In conclusion, NBS programs continue to expand with new conditions due to innovations in both test methods and treatment options. NBS for SCID based on TREC-detection was the first high-throughput DNA technique implemented in screening laboratories. In addition to SCID, there are many other IEI that could benefit from early diagnosis and intervention by preventing severe infections, immune dysregulation and autoimmunity if a suitable NBS test was available. In the next years, the role of KREC analysis, epigenetic immune cell counting, IFN-signatures, protein-profiling and genomic technologies for NBS for IEI will have to be further evaluated in the context of the entire screening process. In addition, other screening criteria and principles including ethical, social, and legal implications, logistics and cost will have to be carefully examined before different IEI can be considered as suitable candidates for inclusion in NBS programs.

Author Contributions

Maartje Blom, Robbert Bredius and Mirjam van der Burg wrote the paper.

Funding

This study was funded by The Netherlands Organisation for Health Research and Development ZonMw (SONNET study, project 543002002; 'Geef XLA geen tijd', project 427002014).

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

1. Fischer, A., Severe combined immunodeficiencies (SCID). Clinical & Experimental Immunology, 2000. **122**(2): p. 143-149.
2. Fischer, A., et al., Severe combined immunodeficiencies and related disorders. Nat Rev Dis Primers, 2015. **1**: p. 15061.
3. Tangye, S.G., et al., Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol, 2020. **40**(1): p. 24-64.
4. Bousfiha, A., et al., Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. Journal of Clinical Immunology, 2020. **40**(1): p. 66-81.
5. Pai, S.Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med, 2014. **371**(5): p. 434-46.
6. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. **130**(25): p. 2718-2727.
7. van Zelm, M.C., et al., PID comes full circle: applications of V(D)J recombination excision circles in research, diagnostics and newborn screening of primary immunodeficiency disorders. Front Immunol, 2011. **2**: p. 12.
8. Hazenberg, M.D., et al., T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. Journal of Molecular Medicine, 2001. **79**(11): p. 631-640.
9. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol, 2005. **115**(2): p. 391-8.
10. Notarangelo, L.D., et al., Human inborn errors of immunity: An expanding universe. Sci Immunol, 2020. **5**(49).
11. Fischer, A., et al., Autoimmune and inflammatory manifestations occur frequently in patients with primary immunodeficiencies. J Allergy Clin Immunol, 2017. **140**(5): p. 1388-1393.e8.
12. Tangye, S.G., et al., The Ever-Increasing Array of Novel Inborn Errors of Immunity: an Interim Update by the IUIS Committee. J Clin Immunol, 2021. **41**(3): p. 666-679.
13. Wilson, J.M. and Y.G. Jungner. Principles and practice of screening for disease. World Health Organization 1968 Oct; 1968/10/01:[Available from: https://apps.who.int/iris/handle/10665/37650.
14. King, J.R., L.D. Notarangelo, and L. Hammarström, An appraisal of the Wilson & Jungner criteria in the context of genomic-based newborn screening for inborn errors of immunity. Journal of Allergy and Clinical Immunology, 2021. **147**(2): p. 428-438.
15. Lankester, A.C., et al., EBMT/ESID inborn errors working party guidelines for hematopoietic stem cell transplantation for inborn errors of immunity. Bone Marrow Transplant, 2021.
16. El-Sayed, Z.A., et al., X-linked agammaglobulinemia (XLA):Phenotype, diagnosis, and therapeutic challenges around the world. World Allergy Organ J, 2019. **12**(3): p. 100018.
17. Tsukada, S., et al., Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell, 1993. **72**(2): p. 279-90.
18. Hagemann, T.L., et al., Genomic organization of the Btk gene and exon scanning for mutations in patients with X-linked agammaglobulinemia. Hum Mol Genet, 1994. **3**(10): p. 1743-9.
19. Vetrie, D., et al., The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature, 1993. **361**(6409): p. 226-33.

20. Abolhassani, H., et al., Mortality and morbidity in patients with X-linked agammaglobulinaemia. Allergol Immunopathol (Madr), 2015. **43**(1): p. 62-6.
21. Chen, X.F., et al., Clinical characteristics and genetic profiles of 174 patients with X-linked agammaglobulinemia: Report from Shanghai, China (2000-2015). Medicine (Baltimore), 2016. **95**(32): p. e4544.
22. Bazregari, S., et al., Evaluation of infectious and non-infectious complications in patients with primary immunodeficiency. Cent Eur J Immunol, 2017. **42**(4): p. 336-341.
23. Bearden, D., et al., Enteroviruses in X-Linked Agammaglobulinemia: Update on Epidemiology and Therapy. J Allergy Clin Immunol Pract, 2016. **4**(6): p. 1059-1065.
24. Winkelstein, J.A., et al., X-linked agammaglobulinemia: report on a United States registry of 201 patients. Medicine (Baltimore), 2006. **85**(4): p. 193-202.
25. Conley, M.E. and V. Howard, Clinical findings leading to the diagnosis of X-linked agammaglobulinemia. J Pediatr, 2002. **141**(4): p. 566-71.
26. Plebani, A., et al., Clinical, immunological, and molecular analysis in a large cohort of patients with X-linked agammaglobulinemia: an Italian multicenter study. Clin Immunol, 2002. **104**(3): p. 221-30.
27. Quinti, I., et al., Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. J Clin Immunol, 2011. **31**(3): p. 315-22.
28. Chun, J.K., et al., Analysis of clinical presentations of Bruton disease: a review of 20 years of accumulated data from pediatric patients at Severance Hospital. Yonsei Med J, 2008. **49**(1): p. 28-36.
29. Serana, F., et al., Use of V(D)J recombination excision circles to identify T- and B-cell defects and to monitor the treatment in primary and acquired immunodeficiencies. J Transl Med, 2013. **11**: p. 119.
30. Borte, S., et al., Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood, 2012. **119**(11): p. 2552-5.
31. Barbaro, M., et al., Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. J Clin Immunol, 2017. **37**(1): p. 51-60.
32. de Felipe, B., et al., Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol, 2016. **27**(1): p. 70-7.
33. Kanegae, M.P.P., et al., NEWBORN SCREENING FOR SEVERE COMBINED IMMUNODEFICIENCIES USING TRECS AND KRECS: SECOND PILOT STUDY IN BRAZIL. Rev Paul Pediatr, 2017. **35**(1): p. 25-32.
34. Trück, J., et al., Swiss newborn screening for severe T and B cell deficiency with a combined TREC/KREC assay - management recommendations. Swiss Med Wkly, 2020. **150**: p. w20254.
35. Nakagawa, N., et al., Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. J Allergy Clin Immunol, 2011. **128**(1): p. 223-225.e2.
36. Health Council of the Netherlands. Neonatal screening: new recommendations. The Hague: Health Council of the Netherlands; 2015. Contract No.: publication no. 2015/08.
37. Baron, U., et al., Epigenetic immune cell counting in human blood samples for immunodiagnostics. Sci Transl Med, 2018. **10**(452).
38. Kalina, T., et al., EuroFlow Standardized Approach to Diagnostic Immunopheneotyping of Severe PID in Newborns and Young Children. Front Immunol, 2020. **11**: p. 371.

39. Hanna, S. and A. Etzioni, MHC class I and II deficiencies. Journal of Allergy and Clinical Immunology, 2014. **134**(2): p. 269-275.
40. Lum, S.H., et al., Hematopoietic Cell Transplantation for MHC Class II Deficiency. Frontiers in Pediatrics, 2019. **7**(516).
41. Cepika, A.-M., et al., Tregopathies: Monogenic diseases resulting in regulatory T-cell deficiency. Journal of Allergy and Clinical Immunology, 2018. **142**(6): p. 1679-1695.
42. Barzaghi, F. and L. Passerini, IPEX Syndrome: Improved Knowledge of Immune Pathogenesis Empowers Diagnosis. Frontiers in pediatrics, 2021. **9**: p. 612760-612760.
43. d'Hennezel, E., et al., FOXP3 forkhead domain mutation and regulatory T cells in the IPEX syndrome. N Engl J Med, 2009. **361**(17): p. 1710-3.
44. Skokowa, J., et al., Severe congenital neutropenias. Nature Reviews Disease Primers, 2017. **3**(1): p. 17032.
45. Fioredda, F., et al., Stem cell transplantation in severe congenital neutropenia: an analysis from the European Society for Blood and Marrow Transplantation. Blood, 2015. **126**(16): p. 1885-92; quiz 1970.
46. Spoor, J., H. Farajifard, and N. Rezaei, Congenital neutropenia and primary immunodeficiency diseases. Critical Reviews in Oncology/Hematology, 2019. **133**: p. 149-162.
47. Maheshwari, A., Neutropenia in the newborn. Current opinion in hematology, 2014. **21**(1): p. 43-49.
48. McNulty, S.N., et al., A Next-Generation Sequencing Test for Severe Congenital Neutropenia: Utility in a Broader Clinicopathologic Spectrum of Disease. J Mol Diagn, 2021. **23**(2): p. 200-211.
49. Livingston, J.H. and Y.J. Crow, Neurologic Phenotypes Associated with Mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, and IFIH1: Aicardi-Goutières Syndrome and Beyond. Neuropediatrics, 2016. **47**(6): p. 355-360.
50. Volpi, S., et al., Type I interferonopathies in pediatric rheumatology. Pediatr Rheumatol Online J, 2016. **14**(1): p. 35.
51. Rice, G.I., et al., Assessment of Type I Interferon Signaling in Pediatric Inflammatory Disease. J Clin Immunol, 2017. **37**(2): p. 123-132.
52. Armangue, T., et al., Neonatal detection of Aicardi Goutières Syndrome by increased C26:0 lysophosphatidylcholine and interferon signature on newborn screening blood spots. Molecular genetics and metabolism, 2017. **122**(3): p. 134-139.
53. Crow, Y.J., J. Shetty, and J.H. Livingston, Treatments in Aicardi-Goutières syndrome. Dev Med Child Neurol, 2020. **62**(1): p. 42-47.
54. Janzi, M., et al., Screening for C3 deficiency in newborns using microarrays. PloS one, 2009. **4**(4): p. e5321-e5321.
55. Hamsten, C., et al., Heat differentiated complement factor profiling. Journal of Proteomics, 2015. **126**: p. 155-162.
56. Dezfouli, M., et al., Newborn Screening for Presymptomatic Diagnosis of Complement and Phagocyte Deficiencies. Frontiers in immunology, 2020. **11**: p. 455-455.
57. Kerfoot, S.A., et al., Tryptic peptide screening for primary immunodeficiency disease by LC/MS-MS. Proteomics Clin Appl, 2012. **6**(7-8): p. 394-402.
58. Massaad, M.J., N. Ramesh, and R.S. Geha, Wiskott-Aldrich syndrome: a comprehensive review. Ann N Y Acad Sci, 2013. **1285**: p. 26-43.
59. Collins, C.J., et al., Rapid Multiplexed Proteomic Screening for Primary Immunodeficiency Disorders From Dried Blood Spots. Frontiers in immunology, 2018. **9**: p. 2756-2756.

60. Collins, C.J., et al., Multiplexed Proteomic Analysis for Diagnosis and Screening of Five Primary Immunodeficiency Disorders From Dried Blood Spots. Front Immunol, 2020. **11**: p. 464.
61. Barben, J., et al., The expansion and performance of national newborn screening programmes for cystic fibrosis in Europe. J Cyst Fibros, 2017. **16**(2): p. 207-213.
62. Bergougnoux, A., M. Lopez, and E. Girodon, The Role of Extended CFTR Gene Sequencing in Newborn Screening for Cystic Fibrosis. International journal of neonatal screening, 2020. **6**(1): p. 23-23.
63. Borte, S., et al., Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. J Allergy Clin Immunol, 2014. **134**(1): p. 226-8.
64. Filipovich, A.H., The expanding spectrum of hemophagocytic lymphohistiocytosis. Curr Opin Allergy Clin Immunol, 2011. **11**(6): p. 512-6.
65. Henter, J.I., et al., HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer, 2007. **48**(2): p. 124-31.
66. Stray-Pedersen, A., et al., Primary immunodeficiency diseases: Genomic approaches delineate heterogeneous Mendelian disorders. Journal of Allergy and Clinical Immunology, 2017. **139**(1): p. 232-245.
67. Pavey, A.R., et al., Utilization of genomic sequencing for population screening of immunodeficiencies in the newborn. Genetics in Medicine, 2017. **19**(12): p. 1367-1375.
68. King, J., J.F. Ludvigsson, and L. Hammarström, Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future. International Journal of Neonatal Screening, 2017. **3**(3): p. 19.
69. King, J.R. and L. Hammarström, Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. Journal of clinical immunology, 2018. **38**(1): p. 56-66.
70. Strand, J., et al., Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol, 2020. **11**: p. 1417.
71. Al-Mousa, H., et al., High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. Front Immunol, 2018. **9**: p. 782.
72. Berg, J.S., et al., Newborn Sequencing in Genomic Medicine and Public Health. Pediatrics, 2017. **139**(2): p. e20162252.
73. Friedman, J.M., et al., Genomic newborn screening: public health policy considerations and recommendations. BMC Med Genomics, 2017. **10**(1): p. 9.
74. Joseph, G., et al., Parental Views on Expanded Newborn Screening Using Whole-Genome Sequencing. Pediatrics, 2016. **137 Suppl 1**(Suppl 1): p. S36-S46.
75. Hasegawa, L.E., et al., Parental attitudes toward ethical and social issues surrounding the expansion of newborn screening using new technologies. Public Health Genomics, 2011. **14**(4-5): p. 298-306.
76. Etchegary, H., et al., Public attitudes about genetic testing in the newborn period. J Obstet Gynecol Neonatal Nurs, 2012. **41**(2): p. 191-200.
77. Almond, B., Genetic profiling of newborns: ethical and social issues. Nat Rev Genet, 2006. **7**(1): p. 67-71.
78. Al-Mousa, H. and B. Al-Saud, Primary Immunodeficiency Diseases in Highly Consanguineous Populations from Middle East and North Africa: Epidemiology, Diagnosis, and Care. Frontiers in immunology, 2017. **8**: p. 678-678.

79. Padilla, C.D., D. Krotoski, and B.L. Therrell, Newborn Screening Progress in Developing Countries—Overcoming Internal Barriers. Seminars in Perinatology, 2010. **34**(2): p. 145-155.
80. El-Sayed, Z.A. and N. Radwan, Newborn Screening for Primary Immunodeficiencies: The Gaps, Challenges, and Outlook for Developing Countries. Frontiers in immunology, 2020. **10**: p. 2987-2987.
81. Blom, M., et al., Second tier testing to reduce the number of non-actionable secondary findings and false positive referrals in newborn screening for severe combined immunodeficiency. Journal of Clinical Immunology, 2021. **Accepted for Publication**.
82. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. **130**(25): p. 2718-2727.
83. Dorsey, M.J., et al., Infections in Infants with SCID: Isolation, Infection Screening, and Prophylaxis in PIDTC Centers. J Clin Immunol, 2021. **41**(1): p. 38-50.
84. EURORDIS. Key Principles for Newborn Screening. 2021 [cited 2021 7 June]; Available from: https://www.eurordis.org/newbornscreening.
85. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunological reviews, 2019. **287**(1): p. 241-252.
86. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. J Allergy Clin Immunol, 2017. **139**(3): p. 733-742.
87. Gutierrez-Mateo, C., et al., Development of a Multiplex Real-Time PCR Assay for the Newborn Screening of SCID, SMA, and XLA. Int J Neonatal Screen, 2019. **5**(4): p. 39.
88. Speckmann, C., et al., Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. J Allergy Clin Immunol, 2012. **130**(4): p. 991-4.
89. la Marca, G., et al., Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency. J Allergy Clin Immunol, 2013. **131**(6): p. 1604-10.
90. la Marca, G., et al., Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots. J Allergy Clin Immunol, 2014. **134**(1): p. 155-9.
91. la Marca, G., et al., The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry. J Pharm Biomed Anal, 2014. **88**: p. 201-6.
92. Malvagia, S., et al., The successful inclusion of ADA SCID in Tuscany expanded newborn screening program. Clin Chem Lab Med, 2021.
93. Thaventhiran, J.E.D., et al., Whole-genome sequencing of a sporadic primary immunodeficiency cohort. Nature, 2020. **583**(7814): p. 90-95.

CHAPTER 12

General discussion

GENERAL DISCUSSION

Severe combined immunodeficiency (SCID) is one of the most severe form of inborn errors immunity (IEI). Patients with SCID have absent or non-functional T-cell (and in some genotypes) B-cells making them suspectable for life-threatening viral, bacterial and fungal infections [1]. Since the case of "the bubble boy' David Vetter in 1971, curative treatment options for SCID have improved significantly. Advances in allogeneic hematopoietic stem cell transplantation (HSCT) have been pioneered in SCID with best outcomes achieved if newborns are treated before onset of infections [2, 3]. Due to the severity of the disease, an asymptomatic status early in life and improved survival and outcome in the absence of pretransplant infections, SCID was considered a suitable candidate for newborn screening (NBS). Since the introduction of the T-cell receptor excision circle (TREC) assay as a sensitive high-throughput test for SCID detection 15 years ago, many countries have introduced SCID in their NBS programs leading to improved outcomes for SCID patients worldwide [3, 4]. NBS programs have shared their experiences, hurdles and challenges associated with the introduction of SCID in their multifaced NBS programs [5-13]. This thesis highlights the many aspects that are associated with NBS for SCID including innovative societal and ethical implications that had never been studied before, as well as technical aspects and cost-effectiveness (Figure 1). This general discussion describes new points of debate, recommendations and future directions coupled with my personal perspective.

Accomplishments of SONNET-study

NBS programs are complex, multi-faceted systems and introduction of SCID could be complicated by unanticipated logistical challenges, a new, relatively expensive screening method, unexpected screening outcomes and a relatively high number of secondary findings. A pilot study would provide the opportunity to evaluate feasibility and disparities prior to national implementation. NBS based on TREC detection has been implemented in many countries with initial pilot studies dating back to 2008 [14, 15]. The aim of our pilot study was therefore not to prove the effectiveness of TREC quantification for the detection of SCID positive cases. However, our pilot study did allow us to introduce a new DNA-based test into the screening laboratory, while training technicians, optimizing laboratory set-up and additionally evaluating cut-off values and screening algorithms all prior to national implementation. In addition, a unique aspect of our pilot was the inclusion of parental perspectives on NBS for SCID emphasizing that implementation of a new condition in a NBS program is not just about introducing a new technique in the lab.

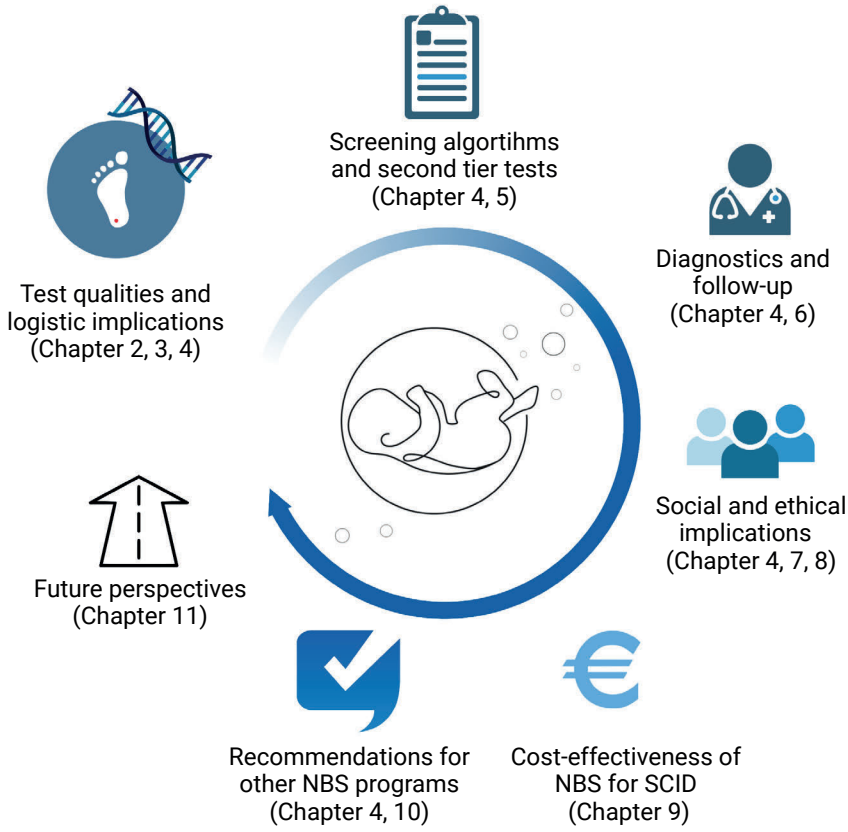

Figure 1. Overview of the different aspects discussed in this thesis

Preparation time for a pilot study should not be underestimated. Prior to our prospective pilot study, we showed with a small-scale proof-of-concept study in **Chapter 2** that measuring TRECS with qPCR could be applied in the Dutch NBS system. In addition, **Chapter 3** described additional preparatory steps ranging from information provision to parents and health care providers to selecting the most suitable TREC assay, laboratory and ICT-adjustments and developing an applicable screening algorithm. A uniform follow-up protocol including a dedicated SCID gene panel for SCID and non-SCID referrals was developed with a multidisciplinary team of experts from all participating academic medical centers. All preparatory steps led to start of the prospective implementation pilot, the SONNET-study (SCID screening Research in the Netherlands with TRECS), on 1 April 2018. The SONNET-study aimed to gather knowledge about the practical implications of NBS for SCID for the Dutch NBS program, while also addressing innovating aspects of NBS for SCID that had never been studied before. This paragraph will focus on the accomplishments of the

SONNET-study and the direct implications for national and international NBS programs as described in **Chapter 4.** Other important aspects of the SONNET-study such as public engagement in NBS programs, ethical dilemmas associated with NBS for SCID and cost-effectiveness will be discussed in the following paragraphs.

The interviews conducted in **Chapter 4** with parents after an abnormal SCID screening gained more in depth insight into their experiences with the referral and follow-up procedure. Their personal experiences showed that the referral procedure needed to be drastically improved as all parents experienced the referral as negative and extremely stressful. Parents stated that they received too little or incorrect information via the general practitioner and would have preferred to be contacted by a pediatrician directly. Questionnaires showed that even parents with a confirmed false-positive result perceived their newborn as more 'vulnerable' implying some effect of going through a referral procedure. The bulletin for general practitioners and parents with key information about NBS for SCID and the SONNET-study did not suffice in these cases. NBS policy is based on a personal relationship of general practitioners with families, who would be able to deliver the news of an abnormal screening result in a comforting setting as a familiar and trusted person for parents. However, we showed that parents were often approached by telephone instead of in person and parents believed the telephone contact to be impersonal and rushed. These findings are most likely not only applicable to the referral procedure for SCID, but should be placed in a broader perspective for other disorders in the NBS program. In my opinion, it cannot be expected from a generalist to have all information about rare conditions included the NBS program, but the need of parents for correct and clear information should be recognized. The most ideal situation would be for a general practitioner to visit the parents at home and deliver the news of an abnormal screening result while being in direct contact with a pediatrician. For some inborn errors of metabolism (IEM) this might not be an option due to the urgent time frame of referral, but for SCID this could definitely be realized. Based on our results in **Chapter 4**, tandem telephone calls with general practitioners and pediatrician-immunologists have now been implemented in the referral procedure after an abnormal TREC result. This way parents can be provided with support and expert information when receiving the news about an abnormal screening result. Even if tandem telephone calls could not be arranged, general practitioners contacted the pediatrician-immunologist prior to contacting parents for additional information and parents were told that they could reach out prior to their appointment in the hospital if they had any urgent questions. In addition to tandem telephone calls, a website with clear and unambiguous information for parents was developed (www.scid.nu).

The results in **Chapter 4** have showed that the majority of parents did not receive or read the written information about the Dutch NBS program or the pilot study. The SONNET-leaflet contained information about SCID, the advantages and disadvantages of participation, privacy regulations and referral procedure. It is debatable if parents can provide informed consent for participation without receiving adequate information. Some parents even declined participation in NBS for SCID due to insufficient information and misconception of the pilot study. The findings in **Chapter 4** have confirmed previous studies stating that NBS education does not always reach parents and there is a persistent lack of public knowledge about NBS [16, 17]. These studies also showed that healthcare providers are the preferred source of NBS information, advocating for incorporation of NBS education into prenatal care and for obstetricians to counsel parents [16, 17]. Information provision and timing of information in NBS has been an ongoing topic of discussion with little consensus between countries [18]. Parents pointed out that the maternity period is a hectic period in which a lot of information is provided. Information leaflets do not seem to serve their purpose and in this digital era, other means such as digital apps or videos should be explored in the near future.

It was already known that uniform diagnostic and clinical follow-up protocols are required for a prompt and consistent approach to definitive diagnosis [19]. Previous studies showed that preventing infections after SCID is diagnosed, remains crucial to impact on survival, highlighting the importance of follow-up protocols with prompt protective measures [3, 20]. Our follow-up protocol was designed in collaboration with all participating academic medical centers to increase uniformity across national centers. This predefined follow-up protocol and SCID gene panel provided guidance to clinical immunologists, pediatric-immunologists and geneticists when dealing with the relatively high number of secondary findings of NBS for SCID. As a result, **Chapter 4** showed that most parents commended their experience with the pediatric-immunologist and were relieved with the rapid availability of diagnostic results. The magnitude of parents' distress while waiting for infants' confirmatory test results should not be underestimated [21]. The follow-up protocol designed for the SONNET-study provided an excellent foundation for the national rollout of follow-up for NBS for SCID to other academic medical centers after national implementation. A close partnership of NBS programs, immunologists, and HSCT specialists in different countries and by sharing experiences internationally could help to improve and promote standardization of follow-up protocols by identifying the best practices.

One of the main accomplishments of the SONNET-study was the detection of the first X-linked SCID patient via NBS in the Netherlands in the asymptomatic phase of the disease [22]. This patient with absent TRECs was referred to the academic

medical center in excellent clinical condition without onset of infections. HSCT with stem cells from 7/10 HLA-matched unrelated cord blood was successful with prompt engraftment and immune reconstitution. Graft-versus-host prophylaxis and *Pneumocystis* prophylaxis were discontinued after swift immunological recovery and the patient is in great condition thus far. The SONNET-study has brought an extraordinary, multidisciplinary team of experts together from varying disciplines to work towards a clear societal goal: national implementation of NBS for SCID in the Netherlands. The success of the SONNET-study can be mainly attributed to the enthusiasm and effort of the close collaboration of NBS laboratory specialists, public health specialists and policy makers, immunologists, laboratory specialist for clinical genetics and pediatric-immunologists. In the end, all findings of the SONNET-study have led to the implementation of SCID in the Dutch NBS program on 1 January 2021. Currently, all five NBS laboratories in the Netherlands are performing the TREC assay on a daily basis and yearly approximately 170,000 newborns are screened for SCID. NBS for SCID in the Netherlands will contribute to improved outcomes of future SCID patients after HSCT: "helping to break the protective bubble in the best possible way".

Patient and public involvement in policymaking

The perspective of parents as key stakeholders in NBS is of great value in policymaking. NBS pilot studies provide an invaluable opportunity to assess parental views on the potential benefits and harms of screening for newborns and their families [23]. Historically, influence of parental perspective on review processes regarding expansion of NBS panels has been limited. In many cases, experts will assume that patients and families will automatically welcome perceived advances in the field. However, this is not necessarily the case and it is important to gauge families perceptions of these advantages. The support of parents in NBS programs is paramount and systematic evaluation of all benefits or harms of adding new conditions is hindered if public opinion is not considered.

Our study was the first to investigate parents' perspectives on NBS for SCID by including an ethical, legal, social implications (ELSI) questionnaire as part of the pilot study. **Chapter 4** investigated the societal and psychosocial aspects of NBS for SCID through the eyes of parents of healthy newborns and parents who received an abnormal SCID screening result for their newborn. NBS for SCID based on TREC quantification had been implemented in several countries, thus the effectiveness of TREC quantification for SCID detection had been demonstrated. However, the availability of a high-quality test method does not automatically guarantee acceptance from the perspective of stakeholders such as parents. Psychosocial aspects had never been reported before in NBS for SCID, therefore our study focused on societal context including public

awareness and parental perspective. Societal acceptance is a major criterion when introducing new disorders in NBS programs, therefore and our questionnaire study amongst parents of healthy newborns showed that parents have a positive attitude towards NBS for SCID. Most parents stated that they wanted SCID to be detected as early as possible for their child. This was in accordance with other studies that also showed public support for expanded NBS and a positive attitude towards NBS in general [16, 24, 25].

Our results in **Chapter 4** specifically focused on NBS SCID, but public and patient involvement should extend beyond NBS to broader health care policy making. Health care policy makers are increasingly recognizing the need to strengthen public involvement and to actively consult and engage the public in health policy decisions. As health care systems aim to promote, restore or maintain the health of a population, the systems should be responsive to the needs of the public [8]. Public and patient involvement will result in more democratic decision making, promoting accountability and demonstrating transparency and openness. In addition, public involvement will help to build trust and public confidence, a key factor in NBS programs [9]. Finally, public opinion can be used to test the suitability of different deliberative techniques to generate evidence for certain policy decisions [2].

Definitions of "the public" usually focus on stakeholders and patient groups, but the general public with differing motivation should be included as important actor. Patient groups are thought to have individual interests based on their diseases, therefore primary focusing on illness experiences. The general population will have a more general and societal interest in healthcare, bringing more general concerns to the discussion. Public opinion should therefore be subcategorized in opinion of general public (the pure public), patients (the affected public), and advocates (experts and interest groups, acting as the partisan public) [26]. In the United States, public opinions can greatly influence NBS policies and problems have arisen from parent group advocacy pressuring individual states to screen for non-recommended disorders. For example, NBS for Krabbe disease, a neurometabolic disease, was introduced after parental group pressure despite opposing opinions from experts [27]. The cost of screening for Krabbe disease was high, the benefit limited and many families were left in uncertainty whether their child was affected or not [28, 29]. Unlike the United States where public opinions can influence NBS policies, advocacy efforts concerning health policy are limited in Europe. Even though patient advocacy groups exist in most European countries, these groups are rarely involved in decisions with regard to expansion of NBS programs [30].

Public involvement with diverse and divergent perspectives will also help formulate culturally appropriate policies, promoting solidarity and collaboration. Cultural and moral believes can be of influence in decision making processes around NBS. When recruiting for public involvement, social diversity including varying demographic characteristics such as age, ethnicity, religion, gender, socio-economic status and levels of education should be taken into account. These characteristics and others may influence perspectives and a broad representation of the population has the potential to produce results that will be widely accepted, as well as enhance respect between diverse groups with varying opinions, and respond to varying needs and concerns [26].

Even though public participation in health policy making is growing, translating outcomes of public engagement to policy remains challenging. Question arise about the form and level of involvement and the relationship with opinions of other stakeholders. Other actors involved in NBS decision making are laboratory scientists, health-care workers, ethical, legal and economic experts, governmental and non-governmental agencies and health-care providers [31]. Policy makers need to balance these different perspectives and needs in discussion about NBS while including considerations about high quality evidence, benefits or harms for the routine screening program, costs, values of the population as well as contextual considerations. For now, with the ever changing landscape of NBS, programs should include a greater diversity of parental opinions by conducting more research on parental preferences and by expanding parental representation on advisory committees.

Secondary findings in NBS for SCID

Every population screening program has to deal with secondary or incidental findings: screen positive findings that are not the target disease and are not intended by the primary aim of the program. A successful screening test has to be extremely sensitive, as missing patients due to false-negative results defeats the purpose of detecting all affected cases. At the same time, screening tests must be highly specific to avoid the referral of a large percentage of the general population for diagnostic testing. From the perspective of health care utilization, down-stream referral centers should not be overrun with false-positive cases and excessive cost. In addition, as described in **Chapter 4,** referrals are associated with significant anxiety and emotional insecurity for parents. Some false-positive are considered an acceptable tradeoff to avoid false-negatives, however, in the Netherlands, the Dutch Health Council believes that secondary findings in a program aimed at screening should be avoided where possible. It is recommended to consistently opt for a test method with the lowest chance of secondary findings, provided multiple tests are available [32]. Secondary findings might occur unexpectedly, but may sometimes already be obvious from the choice of the

test. TRECs are a highly sensitive biomarker for T-cell lymphopenia, but a non-specific marker for the primary target disease SCID, introducing the field of NBS to a palette of neonatal conditions and disorders associated with low T-cells around birth. Several NBS programs have aimed to increase the positive predictive value for SCID by lowering TREC cut-off values, requesting second NBS cards or adjusting screening algorithms [5-13]. Other programs have chosen to include a second tier test after initial TREC analysis such as next generation sequencing (NGS) with gene panels [33, 34]. **Chapter 5** has showed four other potential methods to reduce the number of secondary findings and false-positive referrals in NBS for SCID. Performing second PCR with primers at different positions prevents false-positive referrals caused by TREC region variations leading to primer/probe annealing problems and amplification failure [35]. Epigenetic immune cell counting as a second tier allowed the measurement of relative (epi) $CD3^+$ T-cells counts serving as more direct marker for absolute T-cells in comparison to TRECs [36]. Finally, a lower cut-off value or an adjusted screening algorithm including the distinction between urgent direct referrals and the request for a second NBS could also reduce the number of secondary findings and false-positives cases. Public health programs have a responsibility towards their stakeholders to continuously improve and optimize their NBS programs, therefore all possible adaptations leading to more targeted screening for the core condition and the reduction of false-positive referrals should be explored.

Prior to implementation of the TREC assay, it was already suspected that previously unrecognized conditions will come to light with screening. Neonatal T-cell lymphopenia and low TRECs in trisomy 21, CLOVES syndrome and RAC2 were not associated with neonatal lymphopenia prior to NBS for SCID. Variants in new genes such as BCL11B and EXTL3 were new T-cell deficiencies discovered with whole exome sequencing (WES) and functional analysis after an abnormal TREC result [19]. In **Chapter 6,** we described four cases with profound T-cell lymphopenia due to maternal immunosuppressant use. Some of these infants received prophylaxis during immunological recovery, which raises an interesting question about the follow-up and management of secondary findings to NBS for SCID. One could argue if prophylaxis is really indicated in reversible T-cell defects or are these patients 'overtreated' due to lack of knowledge. On the other hand, prophylaxis will help to bridge the lymphopenic gap, potentially preventing severe infections [37]. There are little studies available on the outcomes of neonatal T-cell lymphopenia due maternal immunosuppressant use. One study showed swift recovery of the T-cell numbers without encountering infections [38], while one study reported severe infections in one patient with fatal outcome [39]. The possibility to a fatal outcome highlights the importance of detecting these cases and in my opinion, which is shared by recent publications, prophylaxis should be considered if profound

T-cell lymphopenia is present [40, 41]. As many countries have included SCID in their NBS program and follow-up strategies differ between countries, more evidence will become available on suitable management for these newborns with T-cell lymphopenia due to maternal immunosuppressant use. Additional data is also required for idiopathic T-cell lymphopenia patients identified with NBS for SCID. These patients have low T-cell numbers, but not low enough to be considered SCID and lack a genetic diagnosis that results in SCID or a known cause of T-cell lymphopenia. T-cell counts may normalize over time, but the timing of this can vary greatly and may take months. In other cases, the T-cell counts remain persistently low or can even decrease over time, which would necessitate consideration of proceeding to HSCT [42, 43]. Follow-up of newborns with idiopathic T-cell lymphopenia will consist of monitoring T-cells numbers by repeated flow cytometry. Prophylaxis in these cases pose an unique management problem as it not clear what constitutes a "protective" T-cell count, or a T-cell count that is sufficient to prevent infections [42]. More data will become available on management of secondary findings in the next years as more countries are adopting SCID screening in their NBS programs. It would therefore be recommended to not only register follow-up data of patients with the target disease, but also include outcome data of patients with other causes for low TRECs. Considering the relatively rarity of these cases, my recommendation would be to combine these international experiences collected by the individual national screening programs.

Actionable versus non-actionable in NBS for SCID

Not all secondary findings are the same, a distinction can be made between three categories: 1) clinically relevant findings; 2) clinical findings (as yet) unclear; and 3) findings that are not clinically relevant. Within the category of clinically relevant findings, further distinction can be made between *actionable findings* where treatment or prevention is possible, and *non-actionable findings* that may be relevant prognostically, but for which no treatment or prevention is available. It can be difficult to make clear statements about actionability.

The ethical dilemma of ataxia telangiectasia in NBS for SCID

Chapter 7 and 8 pose the ethical dilemma of the untreatable disorder ataxia telangiectasia (A-T) as a secondary finding in NBS for SCID. The question remains whether the term *untreatable* is truly in place here and whether A-T should be considered as an *actionable* or *non-actionable* secondary finding. A-T is a rare, autosomal recessively inherited DNA repair disorder leading to a combination of systemic and neurological symptoms, including progressive ataxia, ocular telangiectasias, predisposition to malignancies and a variable immunodeficiency [44]. Patients with classic A-T are asymptomatic in the first year of life, but progressive symptoms will develop shortly

after. A-T is a complex disease to diagnose as clinical presentation and/or laboratory findings vary between patients. A curative treatment for A-T is not yet available, and most patients with the classic form of the disease die before the age of 30 years [44]. While patients with A-T have no curative treatment options, patients are enrolled in specialized care including medical support for pulmonary function, prophylactic antibiotics or immunoglobulins for recurrent infections, and adapted treatment for malignancies [45] The term *untreatable* dismisses the supportive care that is provided for patients and should therefore be avoided. Whether A-T is an actionable or non-actionable secondary finding will be discussed in the section below.

Patients with A-T can present with low TRECs at birth, therefore NBS for SCID can identify some, but not all A-T patients shortly after birth in the pre-symptomatic phase [46-48]. An early diagnosis of A-T would prevent a long diagnostic process with invasive procedures associated with emotional insecurity and anxiety for parents. In addition, as heterozygous carriers of a pathogenic *ATM* mutation have increased risk of developing cancer (especially breast cancer), early diagnosis of the newborn would allow early monitoring and screening for affected family members [48, 49]. In contrast, an early diagnosis of such a severe disorder without curative treatment options would provide a lot of psychological stress in the maturity period and would prevent parents from enjoying the asymptomatic 'golden years'. These advantages and disadvantages of an early diagnosis of A-T were carefully considered by parents of a child with A-T in **Chapter 7** and parents of healthy newborns in **Chapter 8**. In the end, both parents of a child with A-T as well as parents of healthy newborns preferred an early diagnosis in the pre-symptomatic phase of the disease to prevent a long diagnostic process and to ensure optimal clinical guidance from the start.

The discussion about reporting 'untreatable' incidental findings goes hand in hand with the discussion about NBS for 'untreatable' disorders. Several studies including our results in **Chapter 7 and 8** have showed that parents are in favor of addition of childhood-onset disorders to NBS programs, as soon as a valid test is available, regardless of the treatability of the disease [17, 49-51]. According to parents, the traditional aim of NBS programs to identify infants with treatable conditions where early identification prevents irreversible health damage, creates a narrow scope for accessing all benefits of NBS. Parents believe that screening should identify *actionable* conditions including (1) conditions where early interventions lead to health gain for the newborn, (2) conditions where early diagnosis avoids the lengthy diagnostic odyssey and (3) conditions where parents will have reproductive options during subsequent pregnancies [52]. Both parents of children with A-T in **Chapter 7** and parents of healthy newborns in **Chapter 8** included similar arguments when they

expressed their preference for an early A-T diagnosis in the pre-symptomatic phase of the disease. In my perspective, *actionable* should be the preferred term to use in NBS programs and not *treatable*. The benefit for actionable disorders lies in the possibility of managing the disease upon recognizing it early in an infant's life, thus improving health and social outcome for the newborn. However, the definition of actionability in NBS should be limited to the management of the individual affected with the condition. The term actionable should imply that an urgent (early) intervention is required by a specialist and that the intervention results in a demonstrated improvement in outcome. Improvement in outcome or health gain by early diagnosis could also be defined as prevention of comorbidity or improved quality of life (QoL). Long-term outcome studies including QoL studies might be required to determine whether early intervention or diagnosis indeed results in significant health gain.

The prevention of a diagnostic odyssey is an undeniable important aspect of an early diagnosis. An accelerated diagnostic process might prevent excessive invasive diagnostics. In the case of A-T this would entail lumbar punctures, muscle biopsies, and diagnostic X-rays, which are potentially harmful in the context of a DNA-repair disorder. A protracted diagnostic process may affect the psychosocial well-being of the child and its family, while an early diagnosis might also help preparing a family for the condition and its consequences and likely improving the child's well-being. Genetic counseling and being informed about an increased risk for subsequent pregnancies would allow parents to make informed reproductive choices. It would also allow parents to make actionable lifestyle decisions for example living in close proximity to education facilities or healthcare services. However, at this point scientific evidence that these anticipated advantages result in substantial health gain is lacking and the term *actionable* is therefore not yet in place.

Actionable secondary findings in NBS for SCID

The term *actionable* in NBS for SCID can be linked to many of the secondary findings. Neonates with profound T-cell lymphopenia, not meeting all criteria for SCID but eligible for HSCT, would undisputedly be classified as actionable findings. The same would be applicable for patients with complete 22q11.2 deletion syndrome (DiGeorge syndrome), CHARGE syndrome or athymic FOXN1 deficiency, all of which are indications for thymus transplantation [53-55]. Cases of significant T-cell lymphopenia that might benefit from antibiotic prophylaxis, protective isolation, or avoiding live-attenuated vaccines should also be deemed actionable [19, 37]. This is also the case for patients with A-T: the avoidance of ionizing radiation in diagnostic test, prevention of starting HSCT including a conditioning regime and avoidance life-attenuated vaccines are important to prevent malignancies and occurrence of serious infections

by vaccine-strain organisms [56, 57]. The term *actionable* is therefore more suitable than the term *treatable*, as withholding treatment can be important early intervention leading to improved outcomes. Non-actionable secondary findings may be relevant prognostically, but either effective treatments are not available or health benefits from early diagnosis are limited or uncertain. The aim of population based screening is to prevent morbidity or mortality from the targeted disorders through earlier treatment and with limited harm to unaffected infants. Non-actionable secondary findings and referrals of infants with normal lymphocyte numbers by flow cytometry raise concerns about the harm-benefit ratio of screening, and public health programs justifiably strive to prevent referral of these cases [58].

Some countries have included T-cell lymphopenia in addition to SCID as their primary or secondary target in their NBS program. Non-SCID T-cell lymphopenia cases would therefore no longer be considered as secondary findings to NBS for SCID. In the United States, SCID is the core condition, but T-cell related lymphocyte deficiencies are stated as secondary conditions: disorders that are recommended to be screened for while testing the core disorders on the Recommended Uniform Screening Panel (RUSP) [59]. In Norway, the primary target for NBS is SCID and other severe T-cell deficiencies [33]. Including the term T-cell deficiencies as a primary target poses some challenges as not all disorders with T-cell lymphopenia can be detected with the TREC assay. Combined immunodeficiencies such as ZAP-70 deficiency or MHC class I and II gene expression deficiency, have severely impaired T-cell function but can have normal TREC levels as T-cell development is intact beyond the point of TCR gene recombination [60]. High sensitivity might therefore not always be achieved as newborns with T-cell deficiency can be missed with TREC screening. False-negative cases lower the trust in NBS program and vague target diseases diminish the clarity of the program as a whole and undermine its sound justification. Parents might opt out of participation, whereas the program emphatically aims for a high level of participation to avoid missing very severe actionable conditions at birth. In my opinion, the primary target should be defined as 'SCID and *actionable* T-cell deficiencies with low TRECs'. Adjusting a target definition in a NBS program would be accompanied with some changes. Clear information provision to parents is utmost importance in this process. Disease-specific Information should entail that SCID can be treated with HSCT, but actionable T-cell deficiencies can have other types of treatment. Genetic analysis in follow-up protocols should not only focus on variants for SCID, but also on identifying genetic defects for other actionable T-cell deficiencies in line with the IUIS classification [61, 62]. Prior to genetic analysis, parents should be counseled by a clinical geneticist and pediatrician to inform them about severe disorders without highly effective treatment options such as A-T.

Is screening for SCID cost-effective?

Many healthcare systems today are struggling with financial allocation and how much to invest in new medical products, services and pre- and intervention programs including ever-expanding NBS programs. The Wilson and Jungner criteria already stated in 1968 that the cost of case-finding should be economically balanced in relation to possible expenditure on medical care as a whole [63]. Decision analyses and economic evaluations including cost-effectiveness analyses can help inform policy decisions for NBS programs with regard to healthcare resource allocation. Cost-effectiveness analysis (CEA) assesses two or more alternative courses of action in terms of their costs and benefits. Outcomes of each intervention are usually defined in standardized measures such as quality-adjusted-life years (QALYs). The additional cost per QALY gain or the incremental cost effectiveness ratio can subsequently be compared to a threshold value. Cost-effectiveness thresholds (CET) or the maximum expenditure deemed appropriate for a gain vary greatly between countries [64, 65]. In **Chapter 9** costs varied between €41,300 per QALY to €44,100 per QALY for different screening strategies. In the Netherlands, CET can range from €20,000 to €80,000 per QALY depending on the burden of disease, although these numbers are not specific for prevention programs [66]. From a societal perspective higher cost per QALY are justifiable when severe diseases with high societal impact are involved. Explicit CET can aid decision makers when appraising evidence, but should not be the sole metric as CETs are based on assumptions and can lack empirical basis.

Several countries have performed economic evaluations for NBS for SCID, suggesting that NBS for SCID is cost-effective under a range of assumptions about incidence and costs [67-71]. Our data in **Chapter 9** showed that analysis based on real-life data resulted in higher costs, and consequently in less favorable cost-effectiveness estimates for NBS for SCID than previously published analyses based on hypothetical data [72]. These findings indicate that model assumptions and hypothetical predictions need to be verified with actual experience now that programs are in place. Economic evaluations usually rely on model assumptions as information on long-term outcomes of NBS programs is scarce. Long-term outcomes are of key importance for defining the effectiveness of an intervention and should include outcome parameters such as increased incidence, (event-free) survival, morbidity and late complications and quality of life (QoL) [73]. Accurate collection of data that address a broader societal perspective has been a known problem in economic evaluations. In our previously published CEA, we introduced a societal perspective by including productivity costs into the analysis [72]. However, the perspective could be broadened further by including productivity loss of patients with a poor diagnosis or by including use of informal care. In addition, **Chapter 9** highlights that other relevant aspects such as health gain for patients with

a non-SCID diagnosis identified via NBS for SCID could also lead to cost savings and more favorable cost-effectiveness results. Diagnostics costs for non-SCID cases might also be made in a situation without screening, highlighting the challenges when the number of parameters to consider extends beyond the direct costs of screening. Evaluation of QoL associated with a health state is complicated by methodological challenges such as the lack of validated methods for valuing QoL in young children and the need for proxy responders. There is need for a large-scale study on long-term outcomes of SCID patients after HSCT including societal factors such as QoL, productivity (school/work) and health care use included informal care.

The TREC assay is the first high-throughput DNA based test in the NBS laboratory. It is important to keep in mind that introducing a new technique into a laboratory is associated with high cost for extra equipment, reagents and personnel. The estimated costs for screening in our previously published CEA were much lower than the actual screening costs in **Chapter 9**, while actual real-life diagnostic costs were quite comparable to expert opinion and literature based estimations [72]. Although NBS for SCID required introduction of DNA analysis as a primary screening modality for the first time, DNA technology as a platform in NBS laboratories is not limited to SCID. TRECs can be measured simultaneously with other biomarkers for disorders such as X-linked agammaglobulinemia (XLA) and spinal muscular atrophy (SMA) with multiplex PCR, allowing further expansion of NBS programs [74]. Costs for screening of one disorder would be significantly reduced if the test is adaptable to additional condition, as was observed for tandem mass-spectrometry. Even without multiplex PCR, for example in NBS for congenital CMV, the benefit of adaption of DNA extraction and qPCR technology would share costs for reagents, equipment and personnel resulting in a more favorable cost-effectiveness ratio. In addition, if the primary target of NBS for SCID would be changed to 'SCID and actionable T-cell deficiencies with low TRECs', economic evaluations would need to include the additional benefits and cost savings for identifying actionable T-cell lymphopenia at an early stage. As screening and diagnostic costs would remain unchanged, broadening the primary target disease would additionally result in a more favorable cost-effectiveness ratio.

As a final remark, economic evaluations should be incorporated as part of the continuous optimization of NBS programs. Economic evaluations should not only be used in decision-models of implementation of new disorders, but programs should update economic evaluations on a five-to-ten year basis to reevaluate the program. The explorations on cost-effectiveness assumptions on long-term outcomes in **Chapter 9**, were based on literature and expert opinion. International shared learning and publications on long-term outcomes might significantly improve these assumptions. In time, technical advances

might change screening and diagnostic test options, further improve outcomes after HSCT and new treatment modalities such as gene therapy might come into place. With changing demands and new developments updates of existing economic evaluations and are required for continuous optimization of NBS programs.

Screening versus diagnostics

SCID is the first inborn error of immunity (IEI) included in NBS programs. Screening an entire population for these types of conditions is a radical departure from previous scientific and medical paradigms in the field of immunology. Instead of being centered on a patient with signs and symptoms of disease, NBS applies a simple assay for a biomarker to every infant in a population of whom the vast majority are not affected [19]. There are clear differences between screening and diagnostics. The primary purpose of screening is to detect early disease in a large number of apparently healthy individuals. It is important to realize that screening is not the same as prevention; screening can identify disease in an early phase, but it does not prevent the disease from occurring. Most screening tests are not designed to establish a diagnosis, but rather to signal the potential for a serious condition for which specific follow-up must be initiated. Diagnostic testing, on the other hand, is performed to establish the presence (or absence) of a disease in symptomatic or screen positive individuals (confirmatory test) [75]. The differences between screening and diagnostics are depicted in Figure 2.

With the introduction of SCID in NBS, clinical immunologists needed to develop new understanding of screening as screening programs reflect specific aspects of the local population. In addition, public health experts were unfamiliar with immune disorders and needed to learn about follow-up diagnostics after an abnormal SCID screen result. In clinical practice, patients approach professionals for help, while in screening programs, professionals actively encourage the public to undergo a procedure for the benefit of individual cases and the society. Clinicians have the responsibility to do the best for their patients, while screening programs have to balance the befits of the program against the potential harms. For screening programs, the population is the patient. When SCID screening was introduced, these differences created some tension between the field of population-based screening and the field of clinical immunology and diagnostics. From the clinical immunologist's point of view, any newborn with a disorder in which prompt intervention can prevent morbidity and mortality should be flagged in a NBS program, including secondary findings. NBS programs tend to focus on a primary target, stating that secondary findings should be avoided as much as possible. In addition to differences in perspectives, there is also a gap in language and terminology used between these two disciplines. Screeners and immunologists need to join forces to ensure the most optimal screening program and follow-up diagnostics in order to reach the most optimal

clinical care for patients with SCID. The guidelines in **Chapter 10** that we developed with a team of specialists have tried to bring two audiences together, the NBS community and the clinical immunology community, reducing the tension between these fields and bridging the gaps in language and perspective between these disciplines. Standardization of terminology and uniform registration of screening outcomes will promote international exchange of knowledge and improve NBS programs and follow-up care resulting in better health outcomes for children worldwide

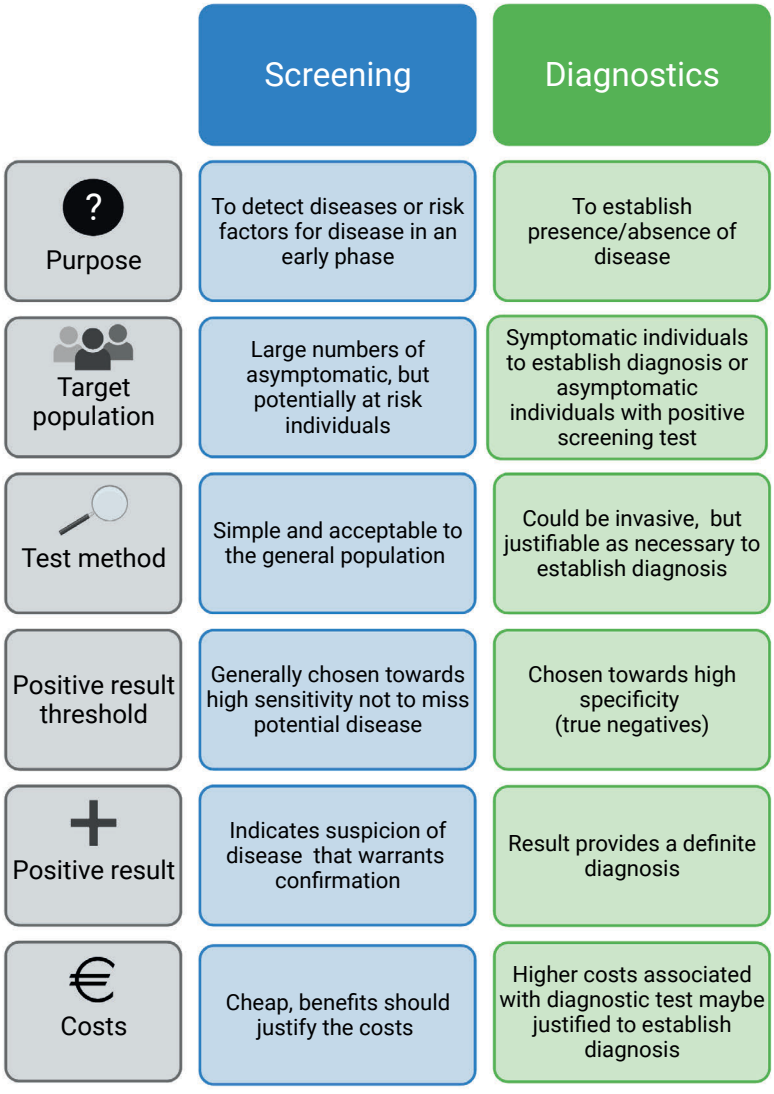

Figure 2. Differences between screening and diagnostic tests

Recommendations and their implementation

This thesis has led to several recommendations specifically for NBS programs that are currently screening for SCID or considering screening for SCID and some more general recommendations applicable to all NBS programs. The recommendations resulting from our experiences in the SONNET-study provided in **Chapter 4** can be expanded with recommendations based on currently available evidence coupled with expert opinion in **Chapter 10** (Figure 3). National NBS programs might benefit most by implementing recommendations that are based on international shared learning including published literature, expert opinion and international guidelines. These type of recommendations can help national NBS programs to optimize their screening algorithms and test modalities to increase the positive predictive value and limit the risk of unnecessary referrals that are associated with high emotional impact for parents and invasive diagnostic testing for the child [58, 76, 77]. As trust in population screening programs is one of the key elements for parents to participate in NBS, international shared learning can help national NBS programs to aim for the highest sensitivity to avoid missing affected children in the direct health interest of the child. Public health programs have a responsibility towards the society as a whole to continuously improve their program, as screening requires resources, and referrals are associated with high diagnostic costs. Implementation of recommendations should therefore be actively pursued by all public health programs worldwide.

Implementing recommendations on an international level might be complicated by the constraints of individual NBS programs. Different countries and even different regions vary greatly in various aspects of NBS programs. These differences can be explained by differing health care organizations, available resources, local politics, professional and patient groups and policy makers [78, 79]. There is little agreement among countries on which disorders should be included in NBS programs, but programs also differ in information provision procedures, time of sample collection, chosen screening tests and screening algorithms, reporting abnormal test results and follow-up protocols [30, 80]. Due to different resources and local regulations even treatment options might differ between countries. In my opinion, it is not necessary to harmonize all preanalytical, analytical and post-analytical aspects of individual programs, but all public health programs have a responsibility towards their stakeholders to continuously improve and optimize their NBS program. Harmonized registration of screening terminology and case definitions as proposed in **Chapter 10** is the first step that needs to be taken in order to recognize opportunities for improvement and to learn from other countries.

It can be somewhat unclear how new recommendations should be implemented in a NBS program and who is responsible for doing so. In my opinion, uniform recommendations should be distributed via coordinating umbrella organizations.

In order to reach a broad public involved in NBS for SCID both organizations for immunologists or clinicians (ESID, CIS) as well as for NBS programs (ISNS, APHL, CLSI) should take the lead. The recommendations for uniform registry of case definitions need to be shared on a broad platform including scientific conferences, newsletters and websites. In the Netherlands, recommendations are usually formulated by groups of experts from different disciplines such as in advisory committees (e.g. ANS-SCID) or the Dutch Health Council. The Ministry of Health, Welfare and Sport will decide which recommendations will be adapted into the NBS program, but the acting organ will be the National Institute for Public Health and the Environment (RIVM). Even on a nation level, implementation of recommendations might take a long time due to the experienced complexity of the program and concerns about disruption of the program in any way. In my opinion, improvements and changes should not be left on hold because of problems that might arise. NBS programs should be flexible and progressive, embracing improvements while staying on top on new developments. If policy makers have concerns about disrupting the program, pilot studies provide a valuable opportunity to introduce improvements or consider new conditions. Stakeholders of different disciplines should be involved when recommendations are implemented into practice. For example, improvements in follow-up protocols cannot be done without the involvement of experts in diagnostics, pediatricians and clinical geneticists, while improvements in referral procedures should be in collaboration with parents and general practitioners.

Using the knowledge and expertise of the stakeholders in the NBS program is of great importance when formulating new recommendations. As **Chapter 10** provides recommendations based on international literature coupled with expert opinions, it would be useful to share these recommendations and definitions with a larger group of experts in the field of immunology and public health through various professional organizations. By distributing these recommendations on a global scale, a broader consensus and input can be obtained prior to implementation. It would therefore be recommended to include a public comment period with a web-based tool to allow other experts to share their opinions and suggestions in a global forum. Finally, the recommendations formulated in **Chapter 10** should be seen as a dynamic and 'living' document, open to suggestions and updating if new insights and experience deem this necessary.

Key messages of this thesis

- Clear information provision by the indicated health care provider both prior to the NBS program as well as during the referral procedure after an abnormal screening result is of utmost importance for parents.
- Uniform follow-up protocols are required for a prompt and consistent approach to achieve a definitive diagnosis and can provide guidance for pediatrician-immunologists when dealing with the relatively high number of incidental findings accompanied by NBS for SCID.
- The term *actionable* is more suited when considering new conditions for NBS programs than the term *treatable*, as withholding treatment can be important early intervention leading to improved outcomes.
- A close partnership of NBS programs, patient organizations, immunologists, geneticists and HSCT specialists in different countries could help to promote standardization of care and follow-up protocols.
- Standardization of terminology in NBS for SCID will bring the screening community and the clinical immunology community together by bridging the gaps in language and perspective between these disciplines.

Recommendations

- Tandem telephone calls by primary health care providers and pediatricians(-immunologists) should be considered when delivering the news about abnormal screening results to parents.
- Follow-up care after an abnormal screening result, independent of the outcome/diagnosis, should be provided as parents can experience (long-term) stress and anxiety after a referral.
- All possible adaptations to the NBS program (including second tier options or adjusted screening algorithms) leading to more targeted screening and the reduction of the number of non-actionable secondary findings and false-positive cases should be explored.
- Parents' perspectives and public involvement should be taken into account when introducing new disorders in NBS programs as societal acceptance is of utmost importance.
- The definition of actionability in NBS should be limited to the management of the individual affected with the condition. The term actionable should imply that an urgent (early) intervention is required by a specialist and that the intervention results in a demonstrated improvement in outcome.
- To improve assumptions and estimations in economic evaluations, a large-scale study on long-term outcomes of SCID patients after HSCT including societal factors such as quality of life, productivity and health care use should be performed.
- Economic evaluations should be updated on five-to-ten year basis for continuous optimization of NBS programs.
- Screening outcomes should be registered in a uniform way in order to promote international exchange of knowledge and improve NBS programs and follow-up care resulting in better health outcomes for children worldwide.

Figure 3. Recommendations and key messages of this thesis.

Future perspectives of NBS for SCID

Expansion of NBS with new disorders is driven by development of new test modalities and treatment options. SCID was the first IEI in population-based screening and at the same time the TREC assay became the first high-throughput DNA-based test in NBS laboratories. In addition to SCID, there are many other IEI that could benefit from early diagnosis and intervention if a suitable NBS test was available. With the Wilson and Jungner screening criteria in mind, several IEI would qualify as serious conditions that cause an important health problem and would benefit from early detection and treatment by preventing severe infections, early onset severe immune dysregulation and auto-immunity [81, 82]. **Chapter 11** describes the advances in technologies such as KREC analysis, epigenetic immune cell counting, protein profiling and genomic techniques such as NGS and whole-genome-sequencing (WGS) that could allow early detection of various IEI shortly after birth. In the next years, the role of these technical advances as well as ethical, social, and legal implications, logistics and cost will have to be carefully examined before different IEI can be considered as suitable candidates for inclusion in NBS programs.

In the near future, SCID will be implemented by an increasing number of NBS programs worldwide. While some NBS program are still awaiting governmental decisions with regard to SCID screening, NBS programs that have already implemented SCID should continue to improve their current practice. Special attention should be paid to follow-up protocol after abnormal screening results as previous studies in the US have showed that many SCID patients identified with NBS still developed infections prior to HSCT [83]. Time required to obtain TREC results, to refer a newborn to a pediatric-immunologist and to obtain results of confirmatory testing should be reduced to prevent significant delay in initiating protective measures. Best practice for isolation and antimicrobial prophylaxis to minimize infection exposure pre-HSCT should be harmonized across centers [84]. In my opinion, in the coming years, NBS programs will acknowledge the importance of early detection of actionable T-cell lymphopenia moving away from SCID screening towards screening for actionable T-cell lymphopenia with low TRECs as the primary target.

In the next five years, many NBS programs will have implemented a multiplex PCR, measuring TRECs simultaneously with KRECs and *SMN1* introducing NBS for XLA and spinal muscular atrophy (SMA) [74]. The addition of KRECs will also be of value to SCID screening as it may assist in distinguishing B-/B+ phenotypes in SCID patients, therefore aiding in the diagnostic process. Some leaky or delayed-onset SCID patients, in particular T-B- SCID patients with hypomorphic mutations in DNA repair or cellular metabolism, might not be detected with TREC quantification [85]. The increase of

toxic metabolites in ADA-SCID patients can well be tolerated to a certain degree by dividing T-cells, whereas B-cells seem to be more vulnerable for genomic stress, for example in patients with delayed-onset ADA-SCID. For these patients, SCID screening will be further extended to tandem mass spectrometry measuring adenosine and deoxyadenosine for ADA deficient patients or purine nucleosides and 2'-deoxy-nucleosides for PNP deficient patients. Previous studies have shown that screening with tandem mass spectrometry was able to identify these infants at low cost [85-88]. The question remains whether PNP patients should be identified via NBS as T-cell numbers are not absent at birth, but progressively decrease over time [61].

After expansion of the tandem mass spectrometry panels, epigenetic immune cell counting will hopefully be optimized as a high-throughput test for the NBS program enabling NBS for a range of IEI. First-tier WGS-based screening in newborns has it's challenges to overcome, but more and more countries will include NGS in their NBS programs as a second tier test in the next decade. As **Chapter 11** describes, at some point, NBS for IEI will enter the genomic era. Genome wide associations studies may have identified an exceeding number of associations between variants and phenotypes. Reference databases will be more complete and pathogenicity prediction programs will demonstrate improved accuracy. Dried blood might no longer be the preferred material for neonatal screening as DNA can be obtained via less invasive techniques such as saliva or oral mucosa. In this era, non-actionable diseases might be included in the NBS program to avoid long diagnostic odysseys. In addition, NBS for early-onset diseases might have been complemented with conditions presenting in adulthood conflicting with the 'child's right to an open future'. Even risk scores of potentially developing a certain disease at some stage in life might be reported early in life. The future of NBS holds many uncertainties, but one thing is sure, with all these technological advances, exciting times are waiting for population-based screening programs.

REFERENCES

1. Fischer, A., Severe combined immunodeficiencies (SCID). Clinical & Experimental Immunology, 2000. 122(2): p. 143-149.
2. Pai, S.Y., et al., Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med, 2014. 371(5): p. 434-46.
3. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
4. Chan, K. and J.M. Puck, Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol, 2005. 115(2): p. 391-8.
5. Amatuni, G.S., et al., Newborn Screening for Severe Combined Immunodeficiency and T-cell Lymphopenia in California, 2010-2017. Pediatrics, 2019. 143(2).
6. Argudo-Ramírez, A., et al., First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain). Frontiers in immunology, 2019. 10: p. 2406-2406.
7. Audrain, M.A.P., et al., Newborn Screening for Severe Combined Immunodeficiency: Analytic and Clinical Performance of the T Cell Receptor Excision Circle Assay in France (DEPISTREC Study). J Clin Immunol, 2018. 38(7): p. 778-786.
8. Zetterström, R.H., et al., Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden. International Journal of Neonatal Screening, 2017. 3(2): p. 11.
9. Kanegae, M.P.P., et al., NEWBORN SCREENING FOR SEVERE COMBINED IMMUNODEFICIENCIES USING TRECS AND KRECS: SECOND PILOT STUDY IN BRAZIL. Rev Paul Pediatr, 2017. 35(1): p. 25-32.
10. Rechavi, E., et al., First Year of Israeli Newborn Screening for Severe Combined Immunodeficiency-Clinical Achievements and Insights. Front Immunol, 2017. 8: p. 1448.
11. Trück, J., et al., Swiss newborn screening for severe T and B cell deficiency with a combined TREC/KREC assay - management recommendations. Swiss Med Wkly, 2020. 150: p. w20254.
12. Hale, J.E., et al., Ten Years of Newborn Screening for Severe Combined Immunodeficiency (SCID) in Massachusetts. J Allergy Clin Immunol Pract, 2021.
13. Routes, J. and J. Verbsky, Newborn Screening for Severe Combined Immunodeficiency. Current Allergy and Asthma Reports, 2018. 18(6): p. 34.
14. Verbsky, J., M. Thakar, and J. Routes, The Wisconsin approach to newborn screening for severe combined immunodeficiency. The Journal of allergy and clinical immunology, 2012. 129(3): p. 622-627.
15. Hale, J.E., et al., Identification of an infant with severe combined immunodeficiency by newborn screening. The Journal of allergy and clinical immunology, 2010. 126(5): p. 1073-1074.
16. DeLuca, J.M., Public Attitudes Toward Expanded Newborn Screening. Journal of pediatric nursing, 2018. 38: p. e19-e23.
17. Hasegawa, L.E., et al., Parental attitudes toward ethical and social issues surrounding the expansion of newborn screening using new technologies. Public Health Genomics, 2011. 14(4-5): p. 298-306.
18. Loeber, J.G., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 1. From blood spot to screening result. Journal of inherited metabolic disease, 2012. 35(4): p. 603-611.

19. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunological reviews, 2019. 287(1): p. 241-252.
20. van der Burg, M., et al., Universal Newborn Screening for Severe Combined Immunodeficiency (SCID). Frontiers in pediatrics, 2019. 7: p. 373-373.
21. DeLuca, J.M., et al., Parents' Experiences of Expanded Newborn Screening Evaluations. Pediatrics, 2011. 128(1): p. 53-61.
22. Blom, M., et al., Voor het eerst Nederlandse SCID patiënt geïdentificeerd met de hielprikscreening. Nederlands Tijdschrift voor Allergie, Astma en Klinische Immunologie, 2021. 1: p. 24-27.
23. Goldenberg, A.J., et al., Including ELSI research questions in newborn screening pilot studies. Genetics in Medicine, 2019. 21(3): p. 525-533.
24. Joseph, G., et al., Parental Views on Expanded Newborn Screening Using Whole-Genome Sequencing. Pediatrics, 2016. 137(Supplement 1): p. S36-S46.
25. Etchegary, H., et al., Interest in newborn genetic testing: a survey of prospective parents and the general public. Genet Test Mol Biomarkers, 2012. 16(5): p. 353-8.
26. Degeling, C., S.M. Carter, and L. Rychetnik, Which public and why deliberate? – A scoping review of public deliberation in public health and health policy research. Social Science & Medicine, 2015. 131: p. 114-121.
27. Wilcken, B. and V. Wiley, Fifty years of newborn screening. Journal of paediatrics and child health, 2015. 51(1): p. 103-107.
28. Kwon, J.M., et al., Consensus guidelines for newborn screening, diagnosis and treatment of infantile Krabbe disease. Orphanet journal of rare diseases, 2018. 13(1): p. 30-30.
29. Orsini, J.J., Newborn screening for Krabbe disease: perceived and current ethical issues. Developmental medicine and child neurology, 2019. 61(12): p. 1354-1354.
30. Burgard, P., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 2. From screening laboratory results to treatment, follow-up and quality assurance. Journal of inherited metabolic disease, 2012. 35(4): p. 613-625.
31. Cornel, M.C., et al., A framework to start the debate on neonatal screening policies in the EU: an Expert Opinion Document. European journal of human genetics : EJHG, 2014. 22(1): p. 12-17.
32. Health Council of the Netherlands, Neonatal screening: new recommendations. 2015, Health Council of the Netherlands: The Hague.
33. Strand, J., et al., Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency. Front Immunol, 2020. 11: p. 1417.
34. Al-Mousa, H., et al., High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. Front Immunol, 2018. 9: p. 782.
35. Vogel, B.H., et al., Newborn screening for SCID in New York State: experience from the first two years. Journal of clinical immunology, 2014. 34(3): p. 289-303.
36. Baron, U., et al., Epigenetic immune cell counting in human blood samples for immunodiagnostics. Sci Transl Med, 2018. 10(452).
37. Dorsey, M.J., et al., Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. J Allergy Clin Immunol, 2017. 139(3): p. 733-742.
38. Coté, C.J., H.J. Meuwissen, and R.J. Pickering, Effects on the neonate of prednisone and azathioprine administered to the mother during pregnancy. J Pediatr, 1974. 85(3): p. 324-8.

39. DeWitte, D.B., et al., Neonatal pancytopenia and severe combined immunodeficiency associated with antenatal administration of azathioprine and prednisone. J Pediatr, 1984. 105(4): p. 625-8.
40. Thomas, C., et al., A Severe Neonatal Lymphopenia Associated With Administration of Azathioprine to the Mother in a Context of Crohn's Disease. Journal of Crohn's and Colitis, 2017. 12(2): p. 258-261.
41. Kuo, C.Y., et al., Profound T-cell lymphopenia associated with prenatal exposure to purine antagonists detected by TREC newborn screening. The Journal of Allergy and Clinical Immunology: In Practice, 2017. 5(1): p. 198-200.
42. Thakar, M.S., et al., A Practical Approach to Newborn Screening for Severe Combined Immunodeficiency Using the T Cell Receptor Excision Circle Assay. Frontiers in immunology, 2017. 8: p. 1470-1470.
43. Albin-Leeds, S., et al., Idiopathic T cell lymphopenia identified in New York State Newborn Screening. Clin Immunol, 2017. 183: p. 36-40.
44. Rothblum-Oviatt, C., et al., Ataxia telangiectasia: a review. Orphanet journal of rare diseases, 2016. 11(1): p. 159-159.
45. van Os, N.J.H., et al., Ataxia-telangiectasia: recommendations for multidisciplinary treatment. Dev Med Child Neurol, 2017. 59(7): p. 680-689.
46. Mallott, J., et al., Newborn screening for SCID identifies patients with ataxia telangiectasia. J Clin Immunol, 2013. 33(3): p. 540-9.
47. Borte, S., et al., Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood, 2012. 119(11): p. 2552-5.
48. Mandola, A.B., et al., Ataxia Telangiectasia Diagnosed on Newborn Screening-Case Cohort of 5 Years' Experience. Front Immunol, 2019. 10: p. 2940.
49. Wood, M.F., et al., Parental attitudes toward newborn screening for Duchenne/Becker muscular dystrophy and spinal muscular atrophy. Muscle Nerve, 2014. 49(6): p. 822-8.
50. Plass, A.M.C., et al., Neonatal Screening for Treatable and Untreatable Disorders: Prospective Parents' Opinions. Pediatrics, 2010. 125(1): p. e99-e106.
51. Hayeems, R.Z., et al., Expectations and values about expanded newborn screening: a public engagement study. Health Expect, 2015. 18(3): p. 419-29.
52. EURORDIS. Key Principles for Newborn Screening. 2021 [cited 2021 7 June]; Available from: https://www.eurordis.org/newbornscreening.
53. Markert, M.L., et al., Thymus transplantation in complete DiGeorge anomaly. Immunol Res, 2009. 44(1-3): p. 61-70.
54. Kreins, A.Y., et al., Correction of both immunodeficiency and hypoparathyroidism by thymus transplantation in complete DiGeorge syndrome. Am J Transplant, 2020. 20(5): p. 1447-1450.
55. Markert, M.L., et al., First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of 2 cases. Blood, 2011. 117(2): p. 688-96.
56. Buchbinder, D., et al., Rubella Virus-Associated Cutaneous Granulomatous Disease: a Unique Complication in Immune-Deficient Patients, Not Limited to DNA Repair Disorders. Journal of Clinical Immunology, 2019. 39(1): p. 81-89.
57. van Os, N.J.H., et al., Diagnosis and Management of Ataxia-Telangiectasia in Resource-Limited Settings. Journal of the International Child Neurology Association, 2020. 1(1).
58. Waisbren, S.E., et al., Effect of Expanded Newborn Screening for Biochemical Genetic Disorders on Child Outcomes and Parental Stress. JAMA, 2003. 290(19): p. 2564-2572.

59. Recommended Uniform Screening Panel. 2018 February 2019 [cited 2020 8 January]; Recommended Uniform Screening Panel]. Available from: https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html.
60. Dorsey, M. and J. Puck, Newborn Screening for Severe Combined Immunodeficiency in the US: Current Status and Approach to Management. Int J Neonatal Screen, 2017. 3(2).
61. Bousfiha, A., et al., Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. J Clin Immunol, 2020. 40(1): p. 66-81.
62. Tangye, S.G., et al., Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol, 2020. 40(1): p. 24-64.
63. Wilson, J.M.G., G. Jungner, and O. World Health, Principles and practice of screening for disease / J. M. G. Wilson, G. Jungner. 1968, World Health Organization: Geneva.
64. Claxton, K., et al., Systematic review of the literature on the cost-effectiveness threshold. Methods for the estimation of the National Institute for Health and Care Excellence cost-effectiveness threshold, 2015.
65. Cameron, D., J. Ubels, and F. Norström, On what basis are medical cost-effectiveness thresholds set? Clashing opinions and an absence of data: a systematic review. Global health action, 2018. 11(1): p. 1447828-1447828.
66. Zwaap, J., et al., Kosteneffectiviteit in de praktijk. Zorginstituut Nederland, 2015.
67. McGhee, S.A., E.R. Stiehm, and E.R. McCabe, Potential costs and benefits of newborn screening for severe combined immunodeficiency. J Pediatr, 2005. 147(5): p. 603-8.
68. Chan, K., et al., A Markov model to analyze cost-effectiveness of screening for severe combined immunodeficiency (SCID). Mol Genet Metab, 2011. 104(3): p. 383-9.
69. Clément, M.C., et al., Systematic neonatal screening for severe combined immunodeficiency and severe T-cell lymphopenia: Analysis of cost-effectiveness based on French real field data. J Allergy Clin Immunol, 2015. 135(6): p. 1589-93.
70. Ding, Y., et al., Cost-Effectiveness/Cost-Benefit Analysis of Newborn Screening for Severe Combined Immune Deficiency in Washington State. J Pediatr, 2016. 172: p. 127-35.
71. Bessey, A., et al., A Cost-Effectiveness Analysis of Newborn Screening for Severe Combined Immunodeficiency in the UK. Int J Neonatal Screen, 2019. 5(3): p. 28.
72. Van der Ploeg, C.P.B et al., Cost-effectiveness of newborn screening for severe combined immunodeficiency. Eur J Ped, 2019. 178(5): p. 721-729.
73. Prosser, L.A., et al., Decision analysis, economic evaluation, and newborn screening: challenges and opportunities. Genetics in medicine : official journal of the American College of Medical Genetics, 2013. 14(8): p. 703-712.
74. Gutierrez-Mateo, C., et al., Development of a Multiplex Real-Time PCR Assay for the Newborn Screening of SCID, SMA, and XLA. Int J Neonatal Screen, 2019. 5(4): p. 39.
75. Gilbert, R., et al., Assessing diagnostic and screening tests: Part 1. Concepts. The Western journal of medicine, 2001. 174(6): p. 405-409.
76. Blom, M., et al., Parents' Perspectives and Societal Acceptance of Implementation of Newborn Screening for SCID in the Netherlands. J Clin Immunol, 2021. 41(1): p. 99-108.
77. Schmidt, J.L., et al., The impact of false-positive newborn screening results on families: a qualitative study. Genet Med, 2012. 14(1): p. 76-80.
78. Martínez-Morillo, E., B. Prieto García, and F.V. Álvarez Menéndez, Challenges for Worldwide Harmonization of Newborn Screening Programs. Clinical Chemistry, 2016. 62(5): p. 689-698.

79. González-Irazabal, Y., G. Hernandez de Abajo, and E. Martínez-Morillo, Identifying and overcoming barriers to harmonize newborn screening programs through consensus strategies. Critical Reviews in Clinical Laboratory Sciences, 2021. 58(1): p. 29-48.
80. Loeber, J.G., et al., Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 1. From blood spot to screening result. J Inherit Metab Dis, 2012. 35(4): p. 603-11.
81. Wilson, J.M. and Y.G. Jungner. Principles and practice of screening for disease. World Health Organization 1968 Oct; 1968/10/01:[Available from: https://apps.who.int/iris/handle/10665/37650.
82. King, J.R., L.D. Notarangelo, and L. Hammarström, An appraisal of the Wilson & Jungner criteria in the context of genomic-based newborn screening for inborn errors of immunity. Journal of Allergy and Clinical Immunology, 2021. 147(2): p. 428-438.
83. Heimall, J., et al., Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood, 2017. 130(25): p. 2718-2727.
84. Dorsey, M.J., et al., Infections in Infants with SCID: Isolation, Infection Screening, and Prophylaxis in PIDTC Centers. J Clin Immunol, 2021. 41(1): p. 38-50.
85. Speckmann, C., et al., Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. J Allergy Clin Immunol, 2012. 130(4): p. 991-4.
86. la Marca, G., et al., Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency. J Allergy Clin Immunol, 2013. 131(6): p. 1604-10.
87. la Marca, G., et al., Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots. J Allergy Clin Immunol, 2014. 134(1): p. 155-9.
88. la Marca, G., et al., The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry. J Pharm Biomed Anal, 2014. 88: p. 201-6.
89. Malvagia, S., et al., The successful inclusion of ADA SCID in Tuscany expanded newborn screening program. Clin Chem Lab Med, 2021.

CHAPTER 13

Summary

SUMMARY

Severe combined immunodeficiency (SCID) is one of the most severe form of inborn errors of immunity (IEI) characterized by the absence or dysfunction of T-lymphocytes affecting both cellular and humoral immunity. Infants with SCID typically appear normal at birth but develop severe infections in the first months of life. Without curative treatment, in the form of allogeneic hematopoietic stem cell transplantation (HSCT) or in some specific forms of SCID, gene therapy (GT), affected infants die within the first year of life. Early definitive treatment, before the onset of infections, results in the best outcomes making SCID a suitable candidate for early detection via newborn screening (NBS). NBS for SCID is based on the detection of T-cell receptor excision circles (TRECs), a marker for thymic production of naïve T-cells. Since the introduction of the TREC assay 15 years ago, many countries have adopted SCID in their NBS programs leading to improved outcomes for SCID patients worldwide. There are several hurdles and challenges associated with the introduction of SCID into these complex, multifaceted NBS programs. This thesis highlights the many aspects that are associated with NBS for SCID addressing innovative societal and ethical implications that had never been studied before, as well as technical aspects and cost-effectiveness within a prospective implementation pilot study. The aim of this thesis was to enable a flawless implementation of NBS for SCID in the Netherlands, while providing valuable recommendations for other countries that are considering SCID screening or for those that want to optimize their implemented NBS SCID program.

Preparations prior to the pilot study

Prior to our prospective pilot study, a small-scale proof-of-concept study was performed in **Chapter 2**. TRECs were retrospectively measured in 1295 NBS cards with a commercially available TREC assay. Preterm newborns showed significantly lower TREC levels compared to full-term newborns and TRECs were absent in peripheral blood spots from 22 confirmed SCID patients. The retest rate would highly depend on the chosen TREC cut-off value. These first experiences with TREC analysis suggested that the TREC measurement with PCR could be adopted into the Dutch screening laboratories. As other TREC assays for SCID became commercially available, an objective comparison study was performed in **Chapter 3** in order to select the most suitable assay for the large-scale prospective pilot study. Based on pre-set objective comparison criteria, the test qualities of both available SCID screening assays were evaluated by measuring 1272 anonymized NBS cards and peripheral blood of 8 SCID patients. Both SCID screening assays turned out to be suitable TREC detecting assays for the Dutch screening laboratories and subtle differences lead to the selection of the assay with the most awarded overall points. **Chapter 3** also illustrated additional

preparatory steps required for the prospective pilot study in the context of the structure of the Dutch NBS program. Information provision for parents and health care providers, adjustments in the laboratory and IT systems, training of technicians and developing an applicable screening algorithm were important aspects to be set in place. A uniform follow-up protocol including a dedicated SCID gene panel for SCID and non-SCID referrals was developed with a multidisciplinary team of experts from all participating academic medical centers.

SONNET-study and the experiences of parents

All preparatory steps led to a prospective implementation pilot called the SONNET-study (SCID screening Onderzoek in Nederland met TRECs) of which the results are discussed in **Chapter 4.** The SONNET-study analyzed 201,470 newborns for SCID from April 2018 to December 2020 in three provinces of the Netherlands (Utrecht, Gelderland and Zuid-Holland). There were 62 newborns with low TREC levels referred for follow-up diagnostics (referral rate of 0.03%). One X-linked SCID patient was identified in the SONNET-study, who underwent successful HSCT with prompt immune reconstitution in with great clinical outcomes thus far. The SONNET-study did not only focus on the technical aspects of NBS for SCID, but also evaluated the perspectives of parents as public uptake and parental acceptance of a test method are not guaranteed. Psychosocial aspects had never been studied before for NBS for SCID and are important for societal acceptance, a major criterion when introducing new disorders in NBS programs. Questionnaires were sent to 2000 parents of healthy newborns who either participated or declined participation in the SONNET-study (response rate 19.6%) The majority of parents of healthy newborns expressed their support for NBS for SCID from both a public health perspective and a personal perspective. Interviews conducted with parents of newborns after abnormal SCID screening results (N = 17) highlighted the emotional impact of an abnormal screening result. Parents experienced significant anxiety and emotional insecurity during the referral procedure highlighting the importance of clear information provision both prior to NBS as well as during the referral procedure. Tandem telephone calls by primary health care providers and pediatrician-immunologists are currently implemented in the Netherlands to deliver the news of an abnormal TREC result. Most parents were relieved with the rapid availability of diagnostic results highlight the importance of uniform follow-up protocols for a prompt and consistent approach to a definitive diagnosis. **Chapter 4** emphasized the importance of parental perspectives when introducing new disorders in NBS programs as they can lead to important recommendations and societal acceptance is of utmost importance.

Secondary findings in NBS for SCID

Even though TRECs are a highly sensitive biomarker for T-cell lymphopenia, they are non-specific for the primary target disease SCID. TREC analysis can identify a range of conditions and disorders associated with low T-cells around birth. NBS programs should continue to optimize their programs aiming for the highest sensitivity while limiting the number of false-positive referrals, as referrals are associated with high emotional impact and anxiety for parents. Different second tier test options and screening strategies in NBS for SCID were therefore evaluated in **Chapter 5.** An alternative quantitative TREC PCR with different primers was performed on NBS cards of referred newborns (N = 56). 45% of the referrals (25/56) had TREC levels above cut-off, including four false-positive cases in which SNPs were identified leading to primer annealing problems and failure of TREC amplification. Epigenetic immune cell counting was used as for relative quantification of $CD3^+$ T-cells in DBS (N = 59). With epigenetic qPCR, 41% (24/59) of the referrals were within the range of the relative $CD3^+$ T-cell counts of the healthy controls. Retrospective data showed that lowering the TREC cut-off value or adjusting the screening algorithm led to lower referral rates but did not prevent all false-positive referrals. **Chapter 5** concluded that second tier tests and adjustments of cut-off values or screening algorithms all have the potential to reduce the number of non-actionable secondary findings in NBS for SCID, although second tier tests are more effective in preventing false-positive referrals.

Some secondary findings were unanticipated in NBS for SCID. **Chapter 6** describes four cases with profound combined T-cell and B-cell lymphopenia due to maternal immunosuppressant identified via NBS for SCID. Both mothers and newborns with reduced TPMT enzyme activity caused by polymorphisms in the *TPMT* gene had less efficient catalyzation of azathioprine leading to a higher risk of hematopoietic toxicity, including profound lymphopenia (mimicking SCID) in the newborn. Two children with reduced TPMT enzyme activity and severe T-cell lymphopenia even required home isolation and initiation of Pneumocystis prophylaxis. All cases demonstrated complete immunological recovery at 10-18 weeks after birth. With the global rollout of NBS for SCID, there is a strong need to raise awareness on a multidisciplinary scale about maternal azathioprine use and the risk of severe neonatal T-cell lymphopenia with abnormal SCID screening results. More explicit monitoring of maternal lymphocyte counts, 6-TGN/6-MMP levels and *TPMT* genotyping at the start of pregnancy, with adjustment of azathioprine dose without reducing therapeutic efficiency in mothers, may prevent fetal exposure to azathioprine toxicity in utero. Moreover, differential blood count analysis in (at-risk) newborns directly after birth may identify these cases prior to NBS for SCID.

Ethical dilemma of uncurable secondary findings

Secondary findings in NBS for SCID can also pose an ethical dilemma such as the early diagnosis of the incurable condition ataxia telangiectasia (A-T). A-T is a severe DNA repair disorder that leads to a broad range of symptoms including neurodegeneration and a variable immunodeficiency. A curative treatment for A-T is not yet available, and most patients with the classic form of the disease die before the age of 30 years. Patients with A-T can be identified with NBS for SCID, leading to an early diagnosis of A-T at birth in a pre-symptomatic stage. **Chapter 7** investigated the opinion of A-T parents on an early A-T diagnosis in the asymptomatic phase of the disease. A questionnaire was sent out to 64 A-T parents (32 families) leading to a response rate of 55%. The majority of parents of a child with A-T (74%) would have preferred an early diagnosis during the asymptomatic phase of the disease, because the uncertainty during the diagnostic process had had a major impact on their lives. In addition, the knowledge of being carriers of an ATM gene mutation influenced decisions about family planning. Parents who opposed against an early diagnosis (14%) emphasized the joy of having a seemingly healthy child until diagnosis. In **Chapter 8** a questionnaire was developed and sent to 4000 parents of healthy newborns who participated in the SONNET-study (response rate 16.6%). The vast majority of parents (81.9%) favoured early diagnosis of A-T over late diagnosis. Main arguments were to avoid a long period of uncertainty prior to diagnosis and to ensure the most optimal clinical care and guidance from the onset of symptoms. Parents who favoured late diagnosis of A-T stated that early diagnosis would not lead to improved quality of life and preferred to enjoy the so-called 'golden years' with their child. The majority of parents (81.1%) stated that they would participate in NBS for A-T if a test was available. Both parents of healthy newborns and parents of A-T patients favored the advantages of early detection of A-T in the asymptomatic phase over the disadvantages, suggesting that first-hand experience with the untreatable disorder is an independent factor in the final opinion of parents on early detection of this disorder.

Cost-effectiveness and NBS for SCID

Cost-effectiveness is key in decision making processes when adding new conditions to a NBS program, especially when screening is associated with a relatively expensive test method as is the case for SCID. **Chapter 9** aimed to refine previous economic evaluations that compared lifetime costs and effects of NBS for SCID to a situation without screening in the Netherlands with real-life data from the SONNET-study. Cost-effectiveness ratios varied from €41,300 per QALY for the screening strategy with TRECs ≤ 6 copies/punch to €44,100 for the screening strategy with a cut-off value of TREC≤ 10 copies/punch. Analysis based on real-life data resulted in higher costs, and consequently in less favorable cost-effectiveness estimates than analyses

based on hypothetical data, indicating the need for verifying model assumptions with real-life data. Comparison of different screening strategies suggested that strategies with a lower number of referrals, e.g., by distinguishing between urgent and less urgent referrals, were favorable from an economic perspective.

Need for uniform case definitions and terminology

Public health programs have the responsibility to continuously optimize their NBS program highlighting the importance of international shared learning. Even though, NBS for SCID has recently been introduced in many countries, comparison of screening outcomes has been hampered by differing terminology among NBS SCID programs. In addition, case definitions for non-SCID conditions with T-cell lymphopenia identified via NBS for SCID are lacking. **Chapter 10** of this thesis highlights the need for standardization of screening terminology to avoid a Babylonian confusion. Improved definitions would promote international exchange of knowledge. A systematic literature review was performed showing the diversity of terminology used in NBS programs internationally. The recommendations in **Chapter 10** reflect currently available evidence and existing guidelines coupled with opinion of experts in public health screening and immunology. While individual screening strategies are expected to be tailored to each program, uniform terminology is lacking when reporting program outcomes. Uniform definitions of disease targets determine sensitivity and specificity and allow comparisons across programs. A distinction was made between actionable and non-actionable T-cell lymphopenia among non-SCID cases. Actionable cases include infants with T-cell lymphopenia who could benefit from antibiotic prophylaxis, protection from infectious exposures or avoiding live-attenuated vaccines. It was proposed that international specialists from each disorder for which NBS is performed join forces to hone their definitions and provide their own recommendations for uniform registration of outcomes of NBS. By bringing together two previously unconnected audiences, the screening community and the clinical immunology community, the guidelines in **Chapter 10** attempt to unite SCID NBS programs by bridging the gaps in language and perspective between these disciplines. Standardization of terminology will promote international exchange of knowledge and optimize each phase of NBS and follow-up care to bring about better health outcomes for children worldwide.

Discussion and future perspectives

In the end, all findings of the SONNET-study presented in this thesis have led to the implementation of SCID in the Dutch NBS program on 1 January 2021. Currently, all five NBS laboratories in the Netherlands are performing the TREC assay on a daily basis and yearly approximately 170,000 newborns are screened for SCID. This thesis has shown the importance of public involvement and evaluation of parental perspective in NBS

policy making. The support of parents in NBS programs is paramount and systematic evaluation of all benefits or harms of adding new conditions is hindered if public opinion is not considered. The importance of uniform follow-up protocol when dealing with secondary findings was discussed with a distinction made between actionable and non-actionable secondary findings. The definition of actionability in NBS should be limited to the management of the individual affected with the condition, implying that an urgent (early) intervention is required by a specialist and that the intervention results in a demonstrated improvement in outcome. Economic evaluations can help inform policy decisions for NBS programs with regard to healthcare resource allocation, but for accurate estimations, a large-scale study on long-term outcomes of SCID patients after HSCT including societal factors such as quality of life, productivity and health care use is needed. The discussion of this thesis highlighted the differences between screening and diagnostics and the gap in language and perspectives between the field of NBS and clinical immunology. Recommendations were presented specifically for NBS programs that are currently screening for SCID or considering screening for SCID and some more general recommendations applicable to all NBS programs. Expansion of NBS with new disorders is driven by development of new test modalities and treatment options. In addition to SCID, other IEI could benefit from early diagnosis and intervention if a suitable NBS test was available. In the future, KREC testing for XLA, epigenetic immune cell counting for quantitative defects of leukocytes and the role of genomic technologies in NBS for IEI will be further explored as described in **Chapter 11**. Even though SCID screening will not be the same 10 years from now due to continuous technological advances, a close partnership of NBS programs' stakeholders, immunologists, geneticists, and pediatricians-immunologists in different countries will be required for moving towards universal SCID screening for all infants. NBS for SCID will contribute to improved outcomes for future SCID patients after HSCT: *helping to break the protective bubble in the best possible way.*

CHAPTER 14

Nederlandse samenvatting
Dutch summary

SAMENVATTING

Severe combined immunodeficiency (SCID) is een zeldzame, ernstige afweerstoornis gekenmerkt door een afwezigheid van functionele T-lymfocyten en daardoor een verstoorde cellulaire en humorale immuniteit. Vlak na de geboorte vertonen kinderen met SCID nog geen symptomen, maar in de eerste levensmaanden ontstaan ernstige, recidiverende infecties, chronische diarree en *failure to thrive*. Zonder behandeling, zoals allogene hematopoëtische stamceltransplantatie (HSCT) of in sommige gevallen autologe hematopoëtische stamcelgentherapie, overlijden kinderen met SCID meestal in het eerste levensjaar. Vroege behandeling, voordat de eerste infecties zijn opgetreden, resulteert in de beste klinische uitkomsten voor de patiënt. Om deze reden zou SCID een geschikte kandidaat zijn voor vroege opsporing via de hielprikscreening. SCID kan worden opgespoord in hielprikbloed door de detectie van *T-cell receptor excision circles* (TRECs). TRECs zijn circulaire, stabiele DNA-fragmenten die gedurende de vroege ontwikkeling van T-lymfocyten ontstaan tijdens de vorming van T-celreceptoren. Kinderen met SCID hebben geen naïeve T-lymfocyten en dus ook geen meetbare TRECs. Op deze manier kunnen SCID patiënten vlak na de geboorte onderscheiden worden van gezonde kinderen. Sinds de introductie van de TREC assay 15 jaar geleden hebben veel landen SCID geïntroduceerd in hun nationale screeningsprogramma's. Het introduceren van een nieuwe aandoening in de complexe structuur van een screeningsprogramma moet echter zorgvuldig gebeuren. Dit proefschrift belicht de vele aspecten die gepaard gaan met neonatale screening op SCID. Zo worden praktische implicaties, kosten en ethische en sociale aspecten van het screenen op SCID in kaart gebracht in een prospectieve implementatie pilotstudie. Het in kaart brengen van de meningen en ervaringen van ouders met betrekking tot SCID screening is nog nooit eerder uitgevoerd en uniek aan dit proefschrift. De resultaten van dit onderzoek zullen leiden tot een optimale nationale implementatie van neonatale screening voor SCID in Nederland, maar dragen ook bij aan belangrijke aanbevelingen voor andere landen die screenen op SCID of implementatie van SCID screening overwegen. Het uiteindelijke doel is het realiseren van geharmoniseerde screening voor SCID en daarmee betere uitkomsten voor SCID patiënten wereldwijd.

Voorbereidingen voor de pilotstudie

Aangezien TREC analyse met PCR een nieuwe analytische techniek is voor de screeningslaboratoria, werd voorafgaand aan onze prospectieve pilotstudie een kleinschalige *proof-of-concept* studie uitgevoerd in **Hoofdstuk 2**. TREC's werden retrospectief gemeten in 1295 hielprikkaartjes met behulp van een commerciële TREC assay. Premature pasgeborenen bleken significant lagere TREC waarden te hebben in vergelijking met voldragen pasgeborenen. Daarnaast waren TRECs afwezig in perifere

bloedvlekken van 22 bevestigde SCID patiënten. Het aantal hertesten zou sterk afhangen van de gekozen TREC afkapwaarde. Deze eerste ervaringen met de TREC-assay suggereerden dat deze techniek geïmplementeerd zou kunnen worden in een Nederlands screeningslaboratorium. Nadat ook andere commerciële TREC assays op de markt kwamen, werden in **Hoofdstuk 3** twee TREC assays met elkaar vergeleken om de meest geschikte assay voor de grootschalige prospectieve pilotstudie te selecteren. De testkwaliteiten van beide SCID screening assays werden geëvalueerd met vooraf opgestelde vergelijkingscriteria door 1272 geanonimiseerde hielprikkaartjes en 8 perifere bloedkaartjes van SCID patiënten te analysen. Beide SCID screening assays bleken geschikt te zijn voor de Nederlandse screeningslaboratoria. De test met de meest toegekende punten werd geselecteerd voor de prospectieve pilot studie. In **Hoofdstuk 3** werden ook andere voorbereidende stappen beschreven. Zo werd informatiemateriaal ontwikkeld voor ouders en stakeholders (screeners, verloskundigen en huisartsen) en werden aanpassingen gedaan in ICT-systemen en screeningslaboratoria. Analisten werden ingewerkt met de nieuwe techniek en er werd een passend screeningsalgoritme ontwikkeld. Tot slot werd door een multidisciplinair team uit alle deelnemende academische medische centra een uniform follow-up protocol ontwikkeld met een SCID genen panel als leidraad voor de genetische analyses na een afwijkend screeningsresultaat.

SONNET-studie en ervaringen van ouders

Alle voorbereidende stappen hebben geleid tot een prospectieve implementatiepilot genaamd de SONNET-studie (SCID screening Onderzoek in Nederland met TRECs) waarvan de resultaten worden besproken in **Hoofdstuk 4.** Van april 2018 tot en met december 2020 zijn er in drie provincies van Nederland (Utrecht, Gelderland en Zuid-Holland) 201.470 kinderen gescreend op SCID. Er werden 62 pasgeborenen met lage TREC waarden verwezen voor vervolgdiagnostiek naar een academisch ziekenhuis (verwijspercentage van 0,03%). Er werd één *X-linked* SCID patiënt geïdentificeerd in de SONNET-studie, die inmiddels succesvol behandeld is met een stamceltransplantatie. Daarnaast werden er andere ernstige oorzaken voor T-lymfocytopenie gevonden die voordeel hadden bij een vroegtijdige diagnose. De SONNET-studie richtte zich niet alleen op de technische aspecten van het screenen op SCID, maar deed ook onderzoek naar de mening en ervaringen van ouders. Psychosociale aspecten waren nog nooit eerder onderzocht voor SCID screening, terwijl publieke betrokkenheid steeds belangrijker is in onderzoek en beleid. Maatschappelijke acceptatie is een belangrijk criterium bij het toevoegen van nieuwe aandoeningen aan hielprikscreeningprogramma's. Er werden vragenlijsten gestuurd naar 2000 ouders van gezonde pasgeborenen (respons 19,6%). De meerderheid van de ouders stond positief tegenover SCID screening, zowel vanuit een maatschappelijk als een persoonlijk perspectief. Uit interviews met ouders van kinderen met een afwijkende SCID screening resultaat (N = 17) bleek dat de emotionele

impact van een verwijzing erg hoog was. De verwijsprocedure werd als negatief ervaren met name door onduidelijke of incorrecte informatie die ouders ontvingen. Daarnaast werd het nieuws van een afwijkend screeningsresultaat op een vervelende manier aan hen gebracht (bijv. via de telefoon of erg gehaast). Deze resultaten benadrukken het belang van duidelijke informatievoorziening zowel voorafgaand aan de hielprikscreening als tijdens de verwijsprocedure. In de huidige verwijsprocedure worden kinderimmunologen daarom telefonisch betrokken bij een verwijzing via de huisarts zodat ouders direct de juiste informatie ontvangen. De meeste ouders waren blij met de snelle uitslag van de diagnostische testen. Een uniform follow-up protocol helpt zorgverleners om tot een snelle definitieve diagnose te komen.

Nevenbevindingen van het screenen op SCID

TRECs zijn een sensitieve biomarker voor T-lymfocytopenie ofwel lage T-cellen, maar ze zijn daardoor niet specifiek voor de primaire doelziekte SCID. Er zijn verschillende aandoeningen die gepaard kunnen gaan met lage T-cellen en dus lage TRECs bij de geboorte. Dit worden de nevenbevindingen van het screenen op SCID genoemd. Hielprikscreeningsprogramma's moeten streven naar de hoogste sensitiviteit, maar tegelijkertijd dient het aantal fout-positieve verwijzen beperkt te blijven. Fout-positieve verwijzingen gaan namelijk niet alleen gepaard met onnodige zorgkosten en medische handelingen, maar ook met een hoge emotionele impact voor ouders zoals hierboven beschreven. De publieke gezondheidszorg heeft een verantwoordelijkheid ten opzichte van zijn stakeholders om screeningprogramma's continu te verbeteren en onderzoek naar opties om fout-positieve verwijzingen te voorkomen hoort daar zeker bij. Om het aantal fout-positieve verwijzingen in Nederland te verlagen zijn er in **Hoofdstuk 5** verschillende *second-tier* testen en alternatieve screeningstrategieën onderzocht. Met een alternatieve TREC assay met andere primers werden TRECs opnieuw gemeten in hielprikkaartjes van verwezen neonaten (N = 56). Een groot deel van deze verwijzingen (45% ofwel 25/56) had nu TREC waarden boven de afkapwaarde. Hieronder waren vier fout-positieve verwijzingen waarin SNP's werden ontdekt die in de originele TREC assay hebben geleid tot hechtingsproblemen van de primers en waardoor er geen amplificatie kon plaatsvinden. Met *epigenetic immune cell counting* kon het relatieve aantal $CD3^+$ T-cellen bepaald worden in hielprikkaartjes van verwezen patiënten (N = 59). In 41% (24/59) van de verwijzingen bleek het relatieve aantal $CD3^+$ T-cellen te liggen in de range van de gezonde controles. Retrospectieve data liet tot slot zien dat het verlagen van de TREC afkapwaarde of het aanpassen van het screeningsalgoritme wel zou leiden tot lagere aantallen verwijzingen, maar dat er nog steeds fout-positieve verwijzingen zouden plaatsvinden. De conclusie van **Hoofdstuk 5** was dat *second-tier* testen en alternatieve screeningstrategieën allemaal geschikt zijn om het aantal verwijzingen omlaag te brengen, al zijn *second-tier* testen effectiever in het voorkomen van fout-positieve verwijzingen.

Sommige nevenbevindingen van het screenen op SCID waren voorafgaand aan de pilotstudie niet eerder onder behandeling bij de kinderimmunoloog. Zo beschrijft **Hoofdstuk 6** vier pasgeborenen opgespoord via de hielprikscreening met ernstige T- en B-lymfocytopenie als gevolg van maternaal immunosuppressiva (azathioprine) gebruik. Zowel moeders als kinderen met een verminderde TMPT-enzymactiviteit veroorzaakt door polymorfismen in het *TPMT*-gen kunnen azathioprine niet goed afbreken, wat leidt tot hematopoëtische toxiciteit en ernstige lymfocytopenie. Bij twee kinderen met verminderde TPMT-enzymactiviteit en ernstige T-lymfocytopenie was zelfs Pneumocystis-profylaxe geïndiceerd. Alle kinderen waren na 10 tot 18 weken volledig immunologisch hersteld. Nu SCID screening wereldwijd wordt geïmplementeerd, is het van belang dat zorgverleners op de hoogte zijn van het risico op neonatale lymfocytopenie en afwijkende SCID screening resultaten bij maternaal azathioprine gebruik. Monitoring van maternale lymfocytenaantallen, 6-TGN/6-MMP-spiegels en *TPMT*-genotypering aan het begin van de zwagerschap kan foetale azathioprine toxiciteit voorkomen. Daarnaast zou een leukocytendifferentiatie bij pasgeborenen direct na de geboorte deze gevallen kunnen opsporen voor de SCID screening uitslag bekend is.

Ethische dilemma's bij ongeneselijke nevenbevindingen

Nevenbevindingen van het screenen op SCID kunnen ook ethische dilemma's met zich meebrengen zoals de vroege diagnose van de ongeneeslijke aandoening ataxia telangiectasia (A-T). A-T is een ernstige DNA-repair stoornis die leidt tot een breed scala aan symptomen, waaronder neurodegeneratie en een variabele immuundeficiëntie. Er is nog geen curatieve behandeling voor AT en de meeste patiënten met de klassieke vorm van de ziekte overlijden voor de leeftijd van 30 jaar. Patiënten met A-T kunnen als nevenbevinding gevonden worden bij het screenen op SCID. De diagnose kan dan kort na de geboorte gesteld worden wat mogelijke voor- en nadelen heeft. Er is daarom in **Hoofdstuk 7** met een vragenlijstenstudie onderzocht hoe ouders van een kind met A-T denken over vroege A-T-diagnose in de presymptomatische fase van de ziekte. Er werd een vragenlijst gestuurd naar 64 ouders van een (of meerdere) kinderen met A-T (32 gezinnen) wat leidde tot een respons van 55%. De meerderheid van de ouders van een kind met A-T (74%) gaf de voorkeur aan een vroege diagnose, vanwege het lange, onzekere diagnostische traject dat zij hadden doorlopen en de grote impact daarvan op hun leven. Bovendien zou de kennis om drager te zijn van een *ATM*-gen mutatie van invloed zijn op beslissingen over gezinsplanning. Ouders die geen vroege diagnose prefereerden (14%), benadrukten de mooie, onbezorgde tijd van het hebben van een ogenschijnlijk gezond kind tot aan de diagnose: de zogenaamde "*golden years*". In **Hoofdstuk 8** is een vragenlijst over dit ethische dilemma verstuurd naar 4000 ouders van gezonde pasgeborenen die deelnamen aan de SONNET-studie

(respons 16,6%). De meerderheid van deze ouders (81,9%) gaf ook de voorkeur aan een vroege diagnose van A-T boven een late diagnose. De belangrijkste argumenten waren het voorkomen van een lang, onzeker diagnostisch traject en het krijgen van de meest optimale klinische zorg en begeleiding wanneer symptomen tot uiting komen. Ouders die de voorkeur gaven aan een latere diagnose van A-T dachten dat een vroege diagnose niet zou leiden tot een betere kwaliteit van leven en wilden liever genieten van de zogenaamde *'golden years'*. De meerderheid van de ouders (81,1%) zou deelnemen aan neonatale screening voor A-T als er een test beschikbaar zou zijn. Zowel de ouders van gezonde pasgeborenen als ouders van A-T patiënten gaven de voorkeur aan een vroege diagnose van A-T in de asymptomatische fase wat impliceert dat ervaring uit de eerste-hand geen bepalende factor was in het uiteindelijke oordeel van ouders. De resultaten van deze vragenlijstenstudies zijn belangrijke bevindingen in de discussie rondom het screenen voor ongeneeslijke aandoeningen via de hielprik.

Kosteneffectiviteit en SCID screening

Kosteneffectiviteit is een belangrijk criterium wanneer nieuwe aandoeningen aan een screeningsprogramma worden toegevoegd. Vooral wanneer screening gepaard gaat met een relatief dure testmethode zoals bij het screenen op SCID. Om deze reden zijn in **Hoofdstuk 9** eerdere economische evaluaties die kosten en effecten van het screenen op SCID vergeleken met een situatie zonder SCID screening in Nederland verfijnd met real-life data uit de SONNET-studie. De kosteneffectiviteitsratio's varieerden van tussen €41.300 per QALY tot €44.100 voor verschillende screeningstrategieën. Real-life data uit de SONNET-studie ging gepaard met hogere kosten en dus minder gunstige schattingen dan op basis van hypothetische data. Dit geeft aan dat modelaannames, ook in kosteneffectiviteitsanalyses uit andere landen, geverifieerd moet worden met data uit pilot studies of geïmplementeerde programma's

Noodzaak van uniforme definities en terminologie

Publieke gezondheidsprogramma's zijn verantwoordelijk voor een optimaal hielprikscreeningsprogramma. Het uitwisselen van kennis tussen landen om verbeterpunten in kaart te kunnen brengen, is hiervoor van groot belang. Hoewel SCID screening inmiddels in verschillende landen is geïntroduceerd, blijkt het onderling vergelijken van resultaten bemoeilijk te worden door de verschillende screeningsterminologie die gebruikt wordt in individuele screeningsprogramma's. Daarnaast zijn er geen uniforme definities van de nevenbevindingen die ook opgespoord worden met het screenen op SCID. **Hoofdstuk 10** van dit proefschrift beschrijft de noodzaak van standaardisatie van screeningterminologie om verwarring te voorkomen en internationale uitwisseling van kennis te bevorderen. Er werd een systematische literatuurstudie uitgevoerd om een overzicht te creëren van de

verschillende termen gebruikt in internationale screeningsprogramma's. Er werd daarnaast een lijst met aanbevelingen opgesteld voor uniforme definities door een groep van internationale experts op het gebied van screening en klinische immunologie. De aanbevelingen werden geformuleerd op basis van definities gevonden in het systematische literatuuronderzoek en termen uit bestaande richtlijnen. Individuele screeningsprogramma's hoeven de gebruikte termen in hun programma's niet aan te passen, maar het zou wenselijk zijn om de uniforme termen te gebruiken in publicaties en rapporten. In **Hoofdstuk 10** wordt daarnaast voorgesteld om een onderscheid te maken tussen *actionable* en *non-actionable* T-lymfocytopenie bij het screenen op SCID. Kinderen met een vorm van *actionable* T-lymfocytopenie hebben baat bij een vroege diagnose doordat er (antibiotica) profylaxe of beschermende maatregelen gestart kunnen worden. Daarnaast kan er voorkomen worden dat deze kinderen een levend verzwakt vaccins krijgen. Internationale experts van andere aandoeningen in het hielprikscreeningsprogramma zouden ook aanbevelingen kunnen doen voor uniforme registratie van uitkomsten. Met de aanbevelingen in **Hoofdstuk 10** wordt het screeningsveld en de klinische immunologie dichter bij elkaar gebracht en worden verschillen in jargon en perspectieven overbrugd. Uiteindelijk zal standaardisatie van terminologie internationale uitwisseling van kennis bevorderen wat kan bijdragen aan de optimalisatie van screeningsprogramma's met de beste gezondheidsuitkomsten voor kinderen wereldwijd tot gevolg.

Discussie en toekomstperspectief

Uiteindelijk hebben alle resultaten in dit proefschrift geleid tot de implementatie van SCID in het Nederlandse hielprikscreeningsprogramma op 1 januari 2021. Momenteel voeren alle vijf screeningslaboratoria in Nederland de TREC assay dagelijks uit en worden er jaarlijks ongeveer 170.000 pasgeborenen gescreend op SCID, met de beste uitkomsten voor Nederlandse SCID patiënten tot gevolg. Dit proefschrift heeft het belang aangetoond van publieke betrokkenheid en de mening van ouders in onderzoek en beleidsvorming. Publieke betrokkenheid bevordert transparantie en openheid en draagt daarmee bij aan algeheel vertrouwen, een belangrijke factor voor deelname in populatie screeningprogramma's. De discussie van dit proefschrift geeft aan dat een uniform follow-up protocol kan helpen bij follow-up van nevenbevindingen en dat er een onderscheid gemaakt zou moeten worden tussen *actionale* en non-*actionable* nevenbevindingen. De definitie van *actionable* in de hielprikscreening zou zich moeten beperken tot de behandeling van de pasgeborene met de aandoening. Een aandoening zou als *actionable* beschouwd moeten worden als een dringende (vroegtijdige) interventie vereist is en dat deze interventie resulteert in aangetoonde verbetering van gezondheidsuitkomsten. De discussie bespreekt daarnaast de rol van economische evaluaties bij beleidsbeslissingen in screeningsprogramma's.

Voor nauwkeurigere schatting in economische evaluaties van SCID-screening is een grootschalig onderzoek naar de lange termijn uitkomsten van SCID-patiënten na HSCT nodig. Dit onderzoek zou ook maatschappelijke factoren zoals kwaliteit van leven, productiviteit en zorggebruik moeten includeren. De discussie van dit proefschrift benadrukt nogmaals de verschillen tussen screening en diagnostiek en de kloof in jargon en terminologie tussen het screeningsveld en de klinische immunologie. Er werden tot slot in de discussie algemene aanbevelingen gedaan voor alle hielprikscreeningsprogromma's en meer specifieke aanbevelingen voor screeningsprogramma's die op SCID screenen of die SCID screening overwegen. SCID is de eerste afweerstoornis in de hielprikscreening, maar er zijn naast SCID ook andere afweerstoornissen die baat zouden hebben bij een vroege diagnose en behandeling als er een geschikte screeningstest beschikbaar zou zijn. Met de Wilson & Jungner screeningscriteria in gedachten zou een vroege behandeling van veel afweerstoornissen kunnen leiden tot het voorkomen van ernstige infecties en auto-immuniteit. Daarnaast zijn HSCT, en mogelijk in de toekomst ook gentherapie, geschikte curatieve behandelingen voor veel monogenetische afweerstoornissen. In **Hoofdstuk 11** is beschreven hoe in de toekomst KREC analyse voor XLA, *epigenetic immune cell counting* voor kwantitatieve defecten van leukocyten en de rol van genomische technologieën in neonatale screening voor afweerstoornissen verder onderzocht zullen worden. Door technologische ontwikkelingen zal het screenen op SCID over 10 jaar niet meer hetzelfde zijn als nu. Een nauwe internationale samenwerking tussen screeningsprogramma's, beleidsmedewerkers, immunologen, genetici en kinderarts-immunologen staat centraal om toe te werken naar universele SCID screening voor alle pasgeborenen wereldwijd, met de beste uitkomsten voor alle toekomstige SCID patiënten tot gevolg.

APPENDIX

List of publications

LIST OF PUBLICATIONS
INTERNATIONAL PEER-REVIEWED PUBLICATIONS

1. **Blom M**., Bredius, R.G.M., van der Burg, M. (2021) Future perspectives of newborn screening for inborn errors of immunity. *International Journal of Neonatal Screening*, 7(4), 74. https://doi.org/10.3390/ijns7040074

2. **Blom, M.,** Zetterström, R.H., Stray-Pedersen, A., Gilmour, K., Gennery, A.R., Puck, J.M., van der Burg, M. (2021) Recommendations for uniform definitions used in newborn screening for severe combined immunodeficiency. *Journal of Allergy and Clinical Immunology*, 16;S0091-6749(21)01401-9. https://doi.org/10.1016/j.jaci.2021.08.026

3. van den Akker-van Marle, M.E, **Blom, M**., van der Burg, M., Bredius, R.G.M., van der Ploeg, C.P.B. (2021) Economic evaluation of different screening strategies for severe combined immunodeficiency based on real-life data. *International Journal of Neonatal Screening*, 7(3):60. https://doi.org/10.3390/ijns7030060

4. **Blom, M**., Pico-Knijnenburg, I., van Montfrans J.M., Bredius R.G.M., van der Burg, M., Swen J.J., Berghuis D. (2021) Abnormal newborn screening for SCID after azathioprine exposure in utero: benefits of TPMT genotyping in mother and child. *Journal of Clinical Immunology*, Online ahead of print. https://doi.org/10.1007/s10875-021-01138-9

5. **Blom, M**., Pico-Knijnenburg, I., Imholz, S., Vissers, L.T.W., Schulze, J.J., Werner, J., Bredius, R.G.M., van der Burg, M. (2021). Second tier testing to reduce the number of non-actionable secondary findings and false-positive referrals in newborn screening for severe combined immunodeficiency. *Journal of Clinical Immunology*, 41 (8), 1762-1773. https://doi.org/10.1007/s10875-021-01107-2

6. Van Dijk, T., Kater, A., Jansen, M., Dondorp, W.J., **Blom, M**., Kemp, S., Langeveld, M., Cornel, M.C., van der Pal, S.M., Henneman, L. (2021). Expanding neonatal bloodspot screening: a multi-stakeholder perspective. *Frontiers in Pediatrics*, 6;9:706394. https://doi.org/10.3389/fped.2021.706394

7. **Blom, M.,** Bredius, R. G. M., Jansen, M. E., Weijman, G., Kemper, E. A., Vermont, C. L., Hollink, I. H. I. M., Dik, W. A., van Montfrans, J. M., van Gijn, M. E., Henriet, S. S., van Aerde, K. J., Koole, W., Lankester, A. C., Dekkers, E. H. B. M., Schielen, P. C. J. I., de Vries, M. C., Henneman, L., van der Burg, M., & on behalf of the SONNET Study Group. (2021). Parents' perspectives and societal acceptance of implementation of newborn screening for SCID in the Netherlands. *Journal of Clinical Immunology*, 41, 99–108. https://doi.org/10.1007/s10875-020-00886-4

8. Schoenaker, M. H. D., **Blom, M.,** de Vries, M. C., Weemaes, C. M. R., van der Burg, M., & Willemsen, M. (2020). Early diagnosis of ataxia telangiectasia in the neonatal phase: a parents' perspective. *European Journal of Pediatrics*, 179(2), 251-256. https://doi.org/10.1007/s00431-019-03479-5

9. **Blom, M.,** Schoenaker, M. H. D., Hulst, M., de Vries, M. C., Weemaes, C. M. R., Willemsen, M. A. A. P., Henneman, L., & van der Burg, M. (2019). Dilemma of reporting incidental findings in newborn screening programs for SCID: parents' perspective on ataxia telangiectasia. *Frontiers in Immunology*, 10, 2438-2438. https://doi.org/10.3389/fimmu.2019.02438

10. Elsink, K., van Montfrans, J. M., van Gijn, M. E., **Blom, M.,** van Hagen, P. M., Kuijpers, T. W., & Frederix, G. W. J. (2020). Cost and impact of early diagnosis in primary immunodeficiency disease: A literature review. *Clinical Immunology*, 213, 108359. https://doi.org/10.1016/j.clim.2020.108359

11. Van der Ploeg, C. P. B., **Blom, M.,** Bredius, R. G. M., van der Burg, M., Schielen, P., Verkerk, P. H., & Van den Akker-van Marle, M. E. (2019). Cost-effectiveness of newborn screening for severe combined immunodeficiency. *European Journal of Pediatrics*, 178(5), 721-729. https://doi.org/10.1007/s00431-019-03346-3

12. Kalina, T., Bakardjieva, M., **Blom, M.,** Perez-Andres, M., Barendregt, B., Kanderová, V., Bonroy, C., Philippé, J., Blanco, E., Pico-Knijnenburg, I., Paping, J., Wolska-Kuśnierz, B., Pac, M., Tkazcyk, J., Haerynck, F., Akar, H. H., Formánková, R., Freiberger, T., Svatoň, M., Šedivá, A., Arriba-Méndez, S., Orfao, A., van Dongen, J. J. M., & van der Burg, M. (2020). EuroFlow standardized approach to diagnostic immunopheneotyping of severe PID in newborns and young children. *Frontiers in Immunology*, 11, 371. https://doi.org/10.3389/fimmu.2020.00371

13. **Blom, M.,** Bredius, R. G. M., Weijman, G., Dekkers, E. H. B. M., Kemper, E. A., Van den Akker-van Marle, M. E., Van der Ploeg, C. P. B., Van der Burg, M., & Schielen, P. C. J. I. (2018). Introducing newborn screening for severe combined immunodeficiency (SCID) in the Dutch neonatal screening program. *International Journal of Neonatal Screening*, 4(4), 40. https://www.mdpi.com/2409-515X/4/4/40

14. **Blom, M.,** Pico-Knijnenburg, I., Sijne-van Veen, M., Boelen, A., Bredius, R. G. M., van der Burg, M., & Schielen, P. (2017). An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. *Clinical Immunology*, 180, 106-110. https://doi.org/10.1016/j.clim.2017.05.007

NATIONAL PEER-REVIEWED PUBLICATIONS

15. **Blom, M.,** Berghuis, D., Vermont, C.L., Kemper, E.A., Weijman, G., Dekkers, E. H. B. M., Dik, W.A., Hollink, I.H.I.M., Langerak, A.W., Bredius, R.G.M., van der Burg, M., mede namens de onderzoeksgroep van de SONNET-studie (2021). Voor het eerst Nederlandse SCID patiënt geïdentificeerd met de hielprikscreening. *Nederlands Tijdschrift voor Allergie, Astma en Klinische Immunologie*, 1, 24-27.

16. **Blom, M.,** van Zanten, E., Berghuis, D., van der Burg, M., Driessen, G. A., & Bredius, R. G. M. (2018). Late diagnose van een patiënt met 'severe combined immunodeficiency'. *Nederlands Tijdschrift voor Allergie, Astma en Klinische Immunologie*, 18, 16-22.

17. **Blom, M.,** Bredius, R. G. M., Driessen, G. J., van Montfrans, J. M., Kuijpers, T. W., Dekkers, E. H. B. M., Schielen, P. C. J. I., & van der Burg, M. (2018). Introductie van 'severe combined immunodeficiency' in het neonatale hielprikscreeningsprogramma in Nederland. *Nederlands Tijdschrift voor Allergie, Astma en Klinische Immunologie*, 18, 23-29.

APPENDIX

PhD PORTFOLIO

PHD PORTFOLIO

PhD training January 2018 to June 2021	Year	Hours
Mandatory courses		
Basic Methods and Reasoning in Biostatistics	2019	42
PhD Introductory Meeting	2018	5
BROK Course	2018	42
Generic/disciplinary courses		
PhD course: Infection, Immunity and Tolerance	2019	80
PhD Curriculum - TULIPS for Child Health	2020	200
Scientific Writing	2019	10
Invited speaker		
International Society for Neonatal Screening (ISNS) - SCID virtual meeting	2021	8
Webinar GenQA - Focus on Molecular Newborn Screenig	2020	4
International Primary Immunodeficiencies Congress (IPIC), Madrid, Spain	2019	20
UKNSLN Annual Scientific Meeting, Manchester, United Kingdom	2019	8
UKNSLN Annual Scientific Meeting, Manchester, United Kingdom	2018	8
Kinderimmunologisches Arbeitstreffen (KIAT), Leipzig, Germany	2018	8
Congress attendance and poster or oral presentations		
Clinical Immunology Society (CIS) Annual Meeting (poster presentation)	2021	24
European Society for Immunodeficiencies (ESID) 19th Online Meeting (two oral presentations)	2020	24
Dutch Society for Immunology (NVVI) Lunteren Symposium Online	2020	16

A

	Year	Hours
NACGG Meeting (oral presentation), Utrecht, the Netherlands	2019	4
Dutch Society for Immunology (NVVI) Annual Meeting, (oral presentation), Noordwijkerhout, the Netherlands	2019	16
European Society for Immunodeficiencies (ESID) 18th Focused Meeting (double oral presentation), Brussels, Belgium	2019	24
5th Workshop on Diagnostics of Immunodeficiencies, Freiburg, Germany	2019	20
11th ISNS European Regional Meeting (poster presentation), Bratislava, Slovakia	2018	24

Teaching activities and student supervision		
Supervisor student Master Medicine - Scientific Internship	2020-2021	78
Supervisor student Bachelor Medicine - Thesis	2021	8
Supervisor students Minor Clinical Immunology LUMC	2020	8
Lecture Master Infection & Immunity Erasmus MC	2018-2020	8
Supervisor student Bachelor Biomedical Sciences – Scientific research project (SPR)	2020	54
Supervisor student Bachelor Medicine – Critical Appraisal of a Topic (CAT)	2020	28
Lecture Bachelor Medicine LUMC	2019	1
Supervisor student HLO Applied Sciences	2019-2020	84

Grants

Medical Inspirator Price 2020 second price (ZonMw): Stop XLA! / Geef XLA geen tijd!

NTvAAKI Topartikel prijs 2018 'Late diagnosis of an atypical patient with severe combined immunodeficiency'

	Year	Hours
Other		
Member Advisory Committee Neonatal Screening – SCID (ANS-SCID) Committee of the Dutch Society of Pediatrics (NvK)	2021 - present	
Training program for certification in Immunology SMBWO. Dutch Society for Immunology	2020 – present	

APPENDIX

DANKWOORD

‘Je eigen ding doen, hoeveel mensen heb je daarbij nodig’

Loesje

DANKWOORD

Heel veel mensen denk ik. Veel te veel mensen om in dit korte dankwoord te kunnen bedanken. Om deze reden heb ik ervoor gekozen om iedereen via onderstaande QR-code op mijn eigen manier te bedanken. Drieënhalf half jaar, 14 hoofdstukken, ontzettend veel woorden en nu is mijn proefschrift eindelijk af. Ik heb in deze periode zoveel bijzondere mensen leren kennen en zoveel steun gehad vanuit verschillende kanten. Onwijs veel dank aan iedereen waarmee ik heb mogen samenwerken, samen lachen, samen ontspannen en samenzijn.

www.doctorblom.nl/dankwoord

APPENDIX

CURRICULUM VITAE

CURRICULUM VITAE

Maartje Blom was born on the 4th of August 1992 in Oud Gastel, the Netherlands. After completing her secondary education (VWO Gymnasium) at the Gertrudiscollege in Roosendaal, she started studying Biomedical Sciences at the University of Utrecht (Utrecht) in 2010. During her bachelor curriculum she became enthusiastic about translational research and the clinical and societal implications of fundamental studies. After successfully finishing her Bachelor's degree in 2013, she therefore applied for a pre-Master Medicine at the University of Leiden (Leiden). With this pre-Master, she was able to enroll in both the Master Medicine and the Master Biomedical Sciences at the Leiden University Medical Center (Leiden). She completed her medical internships at various hospitals and institutes including the academic hospital in Paramaribo (Suriname). Maartje completed her scientific internships at the National Institute for Public Health and the Environment (RIVM, Bilthoven), engaging in the first exploratory steps on newborn screening (NBS) for severe combined immunodeficiency (SCID). Here she gained an increasing interest in the many facets of newborn screening, after which she accepted the offer to continue her research as a PhD candidate in this field. In 2018, she officially started her PhD career at the Laboratory for Immunology, Department of Pediatrics, Leiden University Medical Center (Leiden) under the supervision of dr. Mirjam van der Burg, dr. Robbert Bredius and prof. dr. Arjan Lankester. Maartje combined her medical internships with her PhD research and obtained her Medical degree in 2019 (cum laude) and her Master of Sciences degree in 2020 (cum laude). Her research focused on newborn screening for inborn errors of immunity and included the coordination of the national prospective pilot study on NBS for SCID (the SONNET-study). The studies presented in this thesis are the result of a close collaboration with national and international experts in the field of pediatrics, public health and immunology. As of September 2021, she started her clinical career as a resident not in training (ANIOS) at the Reinier de Graaf Gasthuis in Delft. Furthermore, she continues her scientific work as a parttime researcher in Leiden, focusing on newborn screening for other inborn errors of immunity and coordinating an international quality of life study for SCID patients in the RECOMB-study (gene therapy for RAG-1 SCID). Maartje currently lives in The Hague with her partner Erik.