SCREENING FOR PRIMARY IMMUNODEFICIENCY DISORDERS (PIDDS) IN NEONATES

EDITED BY: Elham Hossny, Antonio Condino-Neto, Lennart Hammarström and Jolan Eszter Walter

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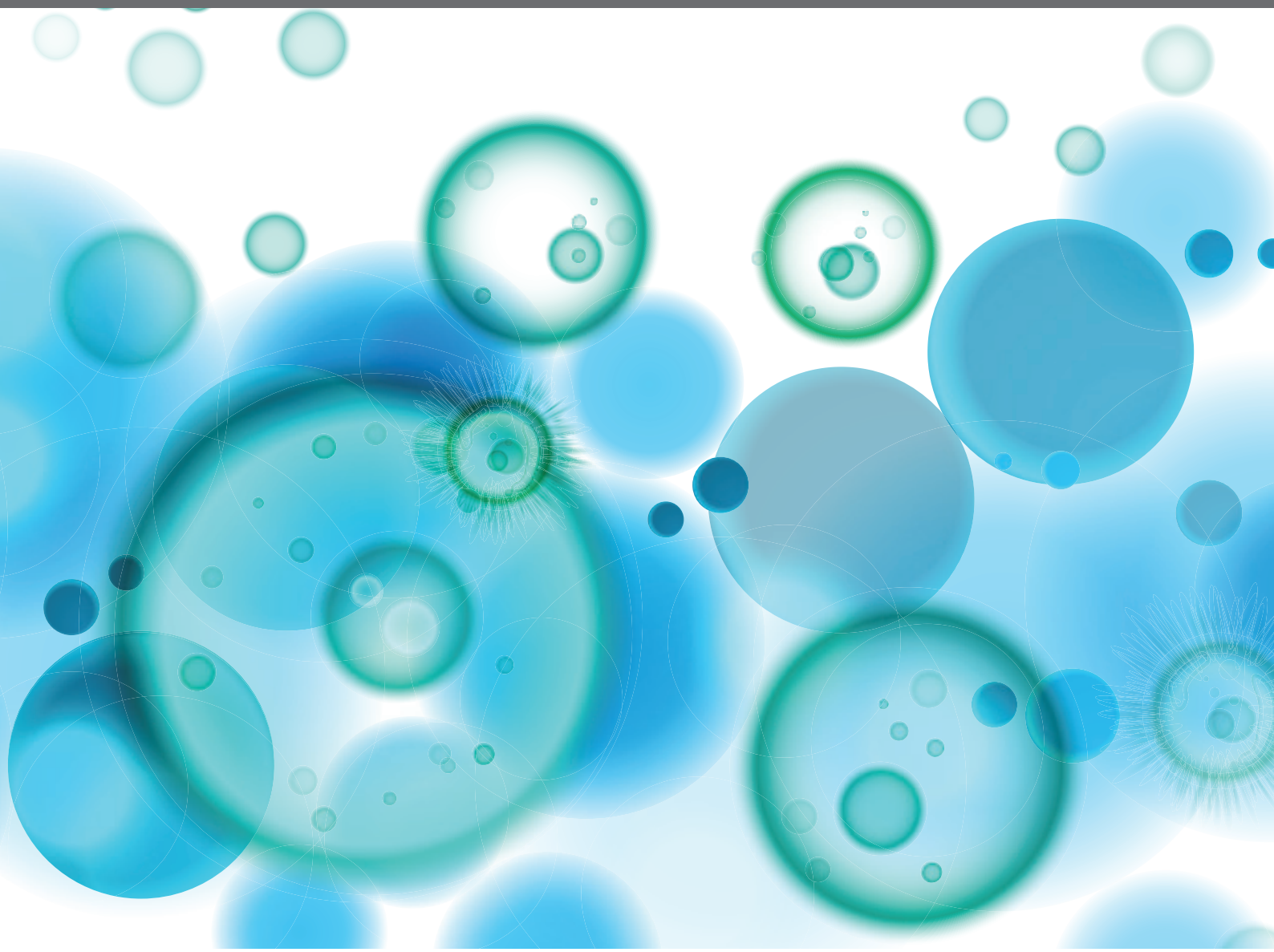

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SCREENING FOR PRIMARY IMMUNODEFICIENCY DISORDERS (PIDDS) IN NEONATES

Topic Editors:
Elham Hossny, Ain Shams University, Egypt
Antonio Condino-Neto, University of São Paulo, Brazil
Lennart Hammarström, Karolinska Institutet (KI), Sweden
Jolan Eszter Walter, University of South Florida, United States

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EDITORIAL
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Editorial: Screening for Primary Immunodeficiency Disorders (PIDDs) in Neonates

Elham Hossny[1]*, *Antonio Condino-Neto*[2], *Lennart Hammarström*[3] *and Jolan Eszter Walter*[4,5,6]

[1] Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt, [2] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [3] Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital, Stockholm, Sweden, [4] Division of Pediatric Allergy and Immunology, University of South Florida, Tampa, FL, United States, [5] Division of Allergy and Immunology, Johns Hopkins All Children's Hospital, St Petersburg, FL, United States, [6] Division of Allergy and Immunology, Massachusetts General Hospital for Children, Boston, MA, United States

Keywords: primary immunodeficiency, screening, newborn, neonates, severe combined immunodeficiency

Editorial on the Research Topic

Screening for Primary Immunodeficiency Disorders (PIDDs) in Neonates

The institution of population-based newborn screening (NBS) has opened a new and exciting era for early diagnosis of primary immunodeficiency disorder (PIDD), which is essential for optimal management of infectious and non-infectious complications leading to high morbidity and mortality. This preventive approach is of high importance for neonates, especially when survival depends on optimal settings for early treatment options such as hematopoietic stem cell transplantation (HSCT). There is an urgent need and opportunity for implementation of neonatal screening for PIDDs globally, as diagnostic tools are developed for SCID and beyond. This Research Topic was aimed to address the current status, limitations and unmet needs of NBS for PIDDs and to draw the attention of immunologists, health care workers and policy makers toward this diagnostic entity in terms of cost effectiveness and global applicability across ethnicities and geographies.

This is a Research Topic collection of 14 articles from several countries around the world. The United States (US) has fully implemented NBS for SCID and now face challenges based on the nationwide variability of the clinical and laboratory approach. Sheller et al. describes the landscape of SCID newborn screening as of 2020, considering the current screening methodologies and targets, communication pathways, and long-term follow-up practices. The authors explore the variation that exists across practices, and emphasize the needs for efficiencies and educational resources in the NBS system to ensure the best outcome. Several papers reported the status of NBS in other geographic locations including data on SCID and other T cell lymphopenia conditions identified among over 130,000 babies screened by T cell receptor excision circles (TRECs) in Catalonia, Spain published by Argudo-Ramírez et al.; and the findings of a group from Hong Kong with Kwok et al., where simultaneous Circles TREC and kappa-deleting recombination excision circles (KREC) quantification were used for detection of a wide variety of PIDD and reference ranges for both KRECs and TRECs were established for distinct age groups. A mini review by El-Sayed and Radwan from Egypt highlights the challenges and major deficits in NBS programs in the developing countries which impedes preventive and curative efforts of managing PIDDs.

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Fabio Candotti,
Centre Hospitalier Universitaire Vaudois (CHUV), Switzerland

***Correspondence:**
Elham Hossny
elham.hossny@gmail.com

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NBS for PIDDs other than SCID is gaining momentum with methods based on multiplex protein profiling from dried blood spot samples for parallel diagnosis of 22 innate immunodeficiencies affecting the complement system and respiratory burst function in phagocytes as published by a multinational group (Dezfouli et al.). The proposed method was validated through retrospective screening of immunodeficient patient samples and is applicable for large population-scale performance. Mandola et al. presented the results of a 5-year cohort for diagnosis of Ataxia Telangiectasia (AT) through NBS in Ontario, Canada. They observed a surprisingly high rate of AT through NBS (one vs. five per year for SCID), with distinct genetic variants and ancestry of the patients. The AT patients detected by NBS displayed more profound immunological and neurological phenotype compared to other AT patients. On the same line, Blom et al. from Netherlands reported on a dilemma about diagnosing AT as incidental finding during NBS for SCID from the parents' perspective. The authors stated that although the current national policy is not to report untreatable incidental findings, unless the health advantage is clear, the majority of parents of healthy neonates in this series were in favor of an early AT diagnosis in the pre-symptomatic phase of the disorder.

Innovations in the methodology of NBS were explored in several articles. Second-tier next generation sequencing (NGS) integrated in the Norwegian nationwide newborn screening program was reported by Strand et al. as means of rapid molecular diagnosis of SCID. Such maneuver on the DNA isolated from the same dried blood spot provided instant confirmation or exclusion of SCID and allowed for the detection of variants of leaky SCID. As a complementary method for SCID-NBS, the multinational European "EuroFlow" standardized approach was proposed by Kalina et al. as unified diagnostic immunopheneotyping for severe PID in children between birth to 2 years of age. The study evaluated the performance of the "SCID-RTE tube" that explores the presence of recent thymic emigrants (RTE) together with T-cell activation status and maturation stages. It was concluded that "EuroFlow SCID-RTE tube" with a previously published PIDOT tube are sensitive and complete cytometric diagnostic test for severe PID (SCID or CID) and for infants identified via NBS with low or absent TRECs. Another multinational study published by Verstegen et al. sought to quantify the T-cell and B-cell replication history in aging, immunodeficiency, and newborn screening. Their results uncovered <5 cell divisions in naive and >10 cell divisions in effector memory T-cell subsets. It also revealed that TREC dilution with age results mainly from increased T cell replication history. Similarly, B cell replication history was higher in patients with primary antibody deficiencies with and without autoimmunity based on KREC assay. The authors propose these assays as second tier to distinguish SCID patients from other PIDs that have false positive NBS for SCID.

Information and emotional support need of families of infants diagnosed as SCID through NBS in the US were explored by Raspa et al. Survey results from parents indicated that the highest-rated information needs were the available treatment options and what to expect across the SCID lifespan. Emotional support needs included dealing with uncertainty about the child's future and additional opportunities to connect with other families.

Three interesting case reports are included in this Research Topic collection. One of them, by Chitty-Lopez et al., reports a novel hypomorphic variant of the recombination-activating gene (RAG) which was identified by newborn screening in an asymptomatic infant with T cell lymphopenia but preserved B cell count and lymphocyte proliferation. This case highlighted how patients with partial RAG deficiency may present with atypical features as identified by NBS for SCID. Confirmatory functional assays and B cell receptor repertoire studies expedited the process that lead to successful HSCT at 5 months of age. In the other case report, Ricci et al. presented the first case of neuroblastoma amplified sequence deficiency (NBAS) disease detected by NBS for SCID via KREC assay. The authors noted that immune dysfunction, which usually takes the form of severe hypogammaglobulinemia, should never go unnoticed in those infants. Lastly, a novel splice site mutation in the interferon gamma receptor-2 (IFNGR2) gene was reported from India by Bandari et al. in patients exhibiting susceptibility to mycobacterial diseases.

The contributors to this special topic highlighted the targets of neonatal screening being not only severe combined immunodeficiency (SCID) but also some other PIDDs. This shows that research in this topic is escalating and lends further evidence for the cost-effectiveness of this life saving approach. It can pave the way for specific strategies to prevent morbidity and mortality in infants from exposure to early life infections including live vaccines. Newborn screening tests are not regular laboratory tests and their establishment in a country mandates caring for other issues including further investigations in suspected infants to confirm the diagnosis and starting pre-transplant care in the form of providing any required medications, encouraging breast feeding when possible, finding a matched donor, avoiding live vaccines and managing complications of BCG vaccination which is compulsory at birth in some countries. It is also mandatory to organize transplant centers' network among countries and provide post-transplant follow up and family counseling facilities. The editors hope that this collection of articles would answer some clinical and investigational queries and might stimulate further research in this domain.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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A Novel Splice Site Mutation in *IFNGR2* in Patients With Primary Immunodeficiency Exhibiting Susceptibility to Mycobacterial Diseases

Aravind K. Bandari[1,2,3†], *Babylakshmi Muthusamy*[1,2,3†], *Sunil Bhat*[4*], *Periyasamy Govindaraj*[5], *Pavithra Rajagopalan*[1], *Aparna Dalvi*[6], *Siddharth Shankar*[3], *Remya Raja*[1,2,3], *Kavita S. Reddy*[1], *Manisha Madkaikar*[6*] and *Akhilesh Pandey*[3,7,8*]

[1] Institute of Bioinformatics, International Technology Park, Bangalore, India, [2] Manipal Academy of Higher Education, Manipal, India, [3] Center for Molecular Medicine, National Institute of Mental Health and Neurosciences, Bangalore, India, [4] Pediatric Haematology, Oncology and Blood & Bone Marrow Transplantation, Mazumdar-Shaw Cancer Center, Narayana Health City, Bangalore, India, [5] Neuromuscular Laboratory, Department of Neuropathology, National Institute of Mental Health and Neurosciences, Bangalore, India, [6] National Institute of Immunohaematology, KEM Hospital Campus, Mumbai, India, [7] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, United States, [8] Center for Individualized Medicine, Mayo Clinic, Rochester, MN, United States

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Edited by:
Antonio Condino-Neto,
University of São Paulo, Brazil

Reviewed by:
Jacinta Bustamante,
Université Paris Descartes, France
Asghar Aghamohammadi,
Tehran University of Medical Sciences, Iran

***Correspondence:**
Sunil Bhat
sunil.bhat.dr@narayanahealth.org
Manisha Madkaikar
madkaikarmanisha@yahoo.co.in
Akhilesh Pandey
pandey.akhilesh@mayo.edu

[†]These authors have contributed equally to this work

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Primary immunodeficiency (PID) refers to a group of heterogeneous genetic disorders with a weakened immune system. Mendelian susceptibility to mycobacterial disease (MSMD) is a subset of PID in which patients exhibit defects in intrinsic and innate immunity. It is a rare congenital disorder characterized by severe and recurrent infections caused by weakly virulent mycobacteria or other environmental mycobacteria. Any delay in definitive diagnosis poses a major concern due to the confounding nature of infections and immune deficiencies. Here, we report the clinical, immunological, and genetic characteristics of two siblings (infants) with recurrent infections. There was a history of death of two other siblings in the family after BCG vaccination. Whole exome sequencing of the two affected surviving infants along with their consanguineous parents identified a novel, homozygous single nucleotide splice acceptor site variant in intron 2 of the interferon gamma receptor 2 (*IFNGR2*) gene. Sanger sequencing of DNA obtained from blood and fibroblasts confirmed the variant. The patients underwent bone marrow transplantation from their father as a donor. RT-PCR and Sanger sequencing of the cDNA of patients from blood samples after transplantation showed the expression of both wild type and mutant transcript expression of *IFNGR2*. To assess partial or complete expression of *IFNGR2* mutant transcripts, fibroblasts were cultured from skin biopsies. RT-PCR and Sanger sequencing of cDNA obtained from patient fibroblasts revealed complete expression of mutant allele and acquisition of a cryptic splice acceptor site in exon 3 that resulted in deletion of 9 nucleotides in exon 3. This led to an in-frame deletion of three amino acids p.(Thr70-Ser72) located in a fibronectin type III (FN3) domain in the extracellular region of IFNGR2. This illustrates individualized medicine enabled by next generation sequencing as identification of this mutation helped in the clinical diagnosis of MSMD in the infants as well as in choosing the most appropriate therapeutic option.

Keywords: *IFNGR2* deficiency, gene therapy, IFN gamma signaling, non-tuberculous mycobacteria, infection, immunodeficiency

INTRODUCTION

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare and genetically heterogeneous immunodeficiency syndrome characterized by predisposition to severe and recurrent infections caused by vaccine against *Mycobacterium tuberculosis* (Bacillus Calmette-Guerin–BCG), which contains weakly virulent non-tuberculous mycobacteria and environmental mycobacteria (1). Predisposition to tuberculosis caused by *Mycobacterium tuberculosis* has also been reported in acquired or inherited immunodeficiencies (2). In addition, patients are susceptible to infections caused by other intracellular bacteria such as listeria and nocardia (3) and fungi such as candida (4), and histoplasma. Finally, viral infections caused by cytomegalovirus, human herpes virus 8 (5), parainfluenza virus type 3, respiratory syncytial virus (6), and varicella zoster virus (7) have also been reported in MSMD.

International Union of Immunological Societies (IUIS) PID expert committee has categorized MSMD as a defect in innate and intrinsic immunity (8). Owing to vaccination practices in many parts of the world, many newborns will receive BCG and those who have MSMD might be recognized as a consequence of this vaccination. The affected individuals have a predisposition to infections which manifests early in childhood and rarely in adulthood. These infections have been reported to affect soft tissues, bone marrow, lungs, skin, bones, and lymph nodes that may or may not recur (8).

The first case report of MSMD was published in 1951 (9) and the first report on its genetic etiology was published in 1996 with autosomal recessive inheritance (10). MSMD exhibits autosomal recessive, autosomal dominant, and X-linked recessive modes of inheritance (1). Mutations in 15 genes are currently known to cause MSMD, which include *IL12RB1*, *IL12B*, *IFNGR1*, *IFNGR2*, *STAT1*, *CYBB*, *IRF8*, *TYK2*, *ISG15*, *RORC*, *IKBKG*, *SPPL2A*, *JAK1*, *IL12RB2,* and *IL23R* (1, 11, 12). Most of these genes were reported with autosomal recessive mode of inheritance whereas *STAT1* was reported with autosomal dominant mode of inheritance. *IFNGR1* and *IRF8* have been reported with both autosomal dominant and autosomal recessive modes of inheritance. Of these genes, *IKBKG,* and *CYBB* are located on the X-chromosome lead to X-linked recessive mode of inheritance (13) (**Table 1**). Gene defects in *IL12RB1* have been reported most frequently followed by *IFNGR1* and *IFNGR2* (1). Approximately 40% of MSMD cases are due to mutations in *IL12RB1* and *IFNGR1* (14). The allelic heterogeneity of MSMD results in partial or complete defects in IFN-γ secretion, production, binding, or signaling (13).

Early detection of infection and accurate diagnosis of MSMD is important for better clinical outcomes. Careful and thorough physical examination and history, especially about consanguinity, and similar complaints in the family are important clues to diagnosing primary immunodeficiency (15). Presence of certain microbial infections throughout the body can further aid diagnosis (15). Laboratory investigations such as blood counts and antibody titers are essential to rule out other causes of immunodeficiency (15). The overall prognosis for MSMD is poor. Patients with MSMD do not generally

TABLE 1 | A list of the currently known 15 genes reported to be associated with Mendelian susceptibility to mycobacterial diseases.

Gene symbol	Protein	Mode of inheritance	Defect
IL12B	Interleukin 12B	AR	Complete deficiency with no mutant protein expression
IL12RB1	Interleukin 12 receptor, beta 1	AR	Complete deficiency with mutant protein expression
		AR	Complete deficiency with no mutant protein expression
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	XR	Partial deficiency with mutant protein expression
IFNGR1	Interferon gamma receptor 1	AR	Complete deficiency and protein expressed
		AR	Complete deficiency and no protein expression
		AD	Partial deficiency and increased protein expression
		AR	Partial deficiency and protein expression
IFNGR2	Interferon gamma receptor 2	AR	Complete deficiency with protein level expression
		AR	Complete deficiency with no protein level expression
		AR	Partial deficiency with protein level expression
		AD	Partial deficiency with protein level expression
STAT1	Signal transducer and activator of transcription 1	AD	Partial deficiency; protein expressed but not phosphorylated
		AD	Partial deficiency; Mutant protein expressed but not bind to DNA
		AD	Partial deficiency; Mutant protein expressed but not phosphorylated or bind to DNA
IRF8	Interferon regulatory factor 8	AD	Partial deficiency with mutant protein expression
CYBB	Cytochrome b-245, beta polypeptide	XR	Complete deficiency with mutant protein expression
ISG15	ISG15 ubiquitin-like modifier	AR	Complete deficiency with no mutant protein expression
RORC	RAR-related orphan receptor gamma	AR	Complete IFNγ deficiency
JAK1	Janus kinase	AR	Complete and partial impaired response to IFNγ
IL12RB2	Interleukin 12 Receptor Subunit Beta 2	AR	Complete IFNγ deficiency with normal/ decreased protein expression
IL23R	Interleukin 23 receptor	AR	Complete IFNγ deficiency with normal/ decreased protein expression
SPPL2A	Signal Peptide Peptidase Like 2A	AR	Complete IFNγ deficiency with no/decreased/normal protein expression

AR, Autosomal recessive; AD, Autosomal dominant; XR, X-linked recessive.
The genetic etiology of these genes such as autosomal recessive (AR), autosomal dominant (AD) or X-linked recessive (XR) and the functional consequences of the mutation whether it is a complete or partial loss of the protein along with its expression are described.

respond to external IFN-γ as they lack functional receptors and hence they survive on antibiotics alone (16). Hematopoietic stem cell transplantation (HSCT) is the only treatment for patients with absent IFN-γ (14, 17, 18). Studies have revealed that the transplants from HLA matched individuals carry a high risk of graft rejection owing to the presence of high IFN-γ concentrations in the plasma of the patients (19, 20).

In this study, we report the clinical, immunological and genetic manifestations of two infants with MSMD born of consanguineous parents. We carried out whole exome sequencing of the proband, his affected brother along with both asymptomatic parents, and identified a novel splice site mutation in *IFNGR2* gene as a potentially genetic cause for MSMD observed in the family. RT-PCR and Sanger sequencing of the cDNA obtained from skin fibroblasts confirmed loss of the conventional splice acceptor site and acquisition of an alternate cryptic splice acceptor site in the exonic region which resulted in an in-frame loss of 9 nucleotides.

MATERIALS AND METHODS

Ethical Statement

This study was carried out in accordance with the recommendations of "ICH-GCP, Indian Council of Medical Research guidelines & Revised Schedule Y Guidelines of Indian Drugs and Cosmetics Rules 1945," Narayana Health Medical Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Narayana Health Medical Ethics Committee.

Exome Sequencing and Data Analysis

Whole exome sequencing and data analysis were carried out as described previously (21). In addition, PROVEAN was used to predict the effect of protein altering indels (22). Further, the shortlisted variants were subjected to segregation analysis using autosomal recessive mode of inheritance which was regarded as the most likely pattern of inheritance based on disease segregation in the family. The shortlisted variants were manually reviewed for potential association of identified mutations in immune related functions. Sanger sequencing of genomic DNA was carried out in a region spanning the splice acceptor site mutation identified in *IFNGR2* gene in patients and their parents. The following are the primers used for sequencing: forward: 5′-CCTCAGCACCCGAAGATTC-3′; reverse: 5′-TTGAAACCAAGGCATTGTCAC -3′.

Transcript Analysis of Blood Samples After Bone Marrow Transplantation

Blood samples were collected in PAXgene blood RNA tubes and total RNA was extracted using the PAXgene blood RNA kit using standard protocols (catalog number # 762164). We used about 1 μg RNA to synthesize single stranded cDNA using High Capacity cDNA Reverse Transcription Kit (Catalog number # 4368814) as per the manufacturer's instructions. In order to validate and study the consequences of the splice site mutation identified in *IFNGR2* gene, we designed primers (F: 5′-GCTCCTCAGCACCCGAAGAT-3′; R: 5′-AGCGATGTCAAAGGGAGAGGA-3′) spanning a region of 405 bp in *IFNGR2* transcript (RefSeq: NM_005534.3). This region is amplified using RT-PCR and the products were run on an agarose gel to visualize the expression of the amplicon. Further, PCR products were cleaned up using QIAquick PCR Purification Kit and the products were quantified and Sanger sequencing was carried out.

Fibroblast Culture and RNA Extraction

Skin biopsy was performed using a 3 mm needle from both the affected siblings. Fibroblasts were grown from the skin biopsies *in vitro* at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified eagle medium (DMEM) with sodium pyruvate and L-glutamine (Gibco #12800-017) and supplemented with sodium bicarbonate, 20% fetal bovine serum, and 1% antibiotic antimycotic solution. Cells were expanded until full confluency and harvested. Total RNA was extracted from the fibroblasts using an RNeasy mini kit. High capacity cDNA reverse transcriptase kit was used for cDNA synthesis for RT-PCR analysis. The PCR products were visualized by agarose gel electrophoresis. Sanger sequencing was performed using the primers described above.

RESULTS AND DISCUSSION

In this study, we recruited an Indian family with two affected siblings born of consanguineous parents. Two other elder siblings in the family died after BCG vaccination. The proband (IV-3) was a third born infant of consanguineous parents (**Figure 1A**). At six months of age, the patient showed symptoms of cervical swellings, abdominal distension, and failure to gain weight. At nine months, he developed fever followed by a persistent cough. BCG vaccination was not administered to this patient because of the adverse effect of BCG observed in the older siblings. The CD4:CD8 ratio was found to be normal with increased CD45 counts. He was normal for a month and after that multiple swellings in the neck, axilla, and groin were observed. The symptoms continued to progress and the child lost weight in spite of a good appetite. There was gradual distension of the abdomen accompanied by dry and scaly skin changes. He was passing stools 4–5 times a day which were watery, non-bulky, non-greasy, and semi-solid. He had severe pallor, dependent edema, and features suggestive of congestive cardiac failure (CCF). Following blood transfusions and management of CCF the patient improved. Child also received nutritional support and multivitamins. Patient had a reactive marrow and therefore, not suggestive of lymphoma, or dimorphic anemia with neutrophilic leucocytosis. Chest X-ray was normal, ultrasound studies of the abdomen showed hepatosplenomegaly with mesenteric lymphadenopathy. Hemoglobin electrophoresis was normal. TORCH, a screening test for toxoplasmosis, others (HIV, hepatitis viruses, varicella, parvovirus), rubella (German measles), cytomegalovirus, and herpes simplex was normal. Lymph node biopsy showed reactive lymphadenitis with some histiocytes. Both TB-PCR and Mantoux tests were negative. Other investigations revealed decreased platelets and increased C-reactive protein (CRP) levels. Evaluation for HLH was

normal, EBV-PCR was negative, and lymph node culture for AFB (BACTEC) revealed no growth. Haplotype-matched bone marrow transplant from the father was performed due to severe, recurring infections in the patient. The patient is stable and is cured of his disease.

The younger sibling (IV-4) of the proband (IV-3) is the fourth affected child in the family (**Figure 1A**). As in the case of IV-3, IV-4 manifested symptoms at 9 months. He had fever and enlarged lymph nodes in the neck. BCG vaccine was not administered owing to the adverse reactions observed in the elder deceased siblings. A haplotype-matched bone marrow transplant from the father was performed and he is also cured of his disease.

The first born (IV-1) was a male infant delivered after a normal pregnancy. He had an adverse reaction to BCG upon vaccine administration. The first infection was an episode of cold and cough followed by left axillary and cervical swelling at 3 months. Because of the prevailing fever, he was hospitalized for 20 days. Abdominal distension and hepatosplenomegaly were observed. The infant passed away at 6 months of age. The second born (IV-2) was a female infant who also had an adverse reaction to BCG vaccine. The infant developed swelling in the left axilla and was diagnosed to have disseminated BCG lymphadenitis. She was suspected to have T-cell deficiency and the lymph nodes were removed; however, the open wound ulcerated and the she died at 4 months of age.

Whole Exome Sequencing and Analysis

In order to identify potential genetic cause for the phenotypes observed in patients, we performed whole exome sequencing of the two surviving infants and their parents. We obtained an average of 64 million reads of which ∼99% were aligned to the human reference genome (hg19). We obtained an average depth of 78× across the four individuals. After post alignment quality measures, we carried out joint variant calling across the four members. We obtained a total of 1,292,418 variants. Gene annotation of these variants using Annovar resulted in the identification of 45,817 exonic and 341 splice site variants, which were considered for further downstream analysis. Common variants with minor allele frequency >0.01 were discarded after comparing the variants with 1,000 genomes project, ExAC, and EVS resulting in 5,932 variants. Filtering variants based on autosomal recessive pattern of inheritance resulted in three variants that corresponded to a missense variant in *SON*, a frameshift causing insertion in *KIR2DL4* (rs11371265), and a novel splice acceptor site variant in *IFNGR2* (**Table 2**; **Figure 1B**). SON is a DNA binding protein involved in splicing and its role in immune related functions is not known.

A single nucleotide insertion c.802dupA resulting in a frame shift in *KIR2DL4* gene (RefSeq: NM_001080772.2) was identified in homozygous state in both patients and heterozygous state in the consanguineous parents. *KIR2DL4* gene encodes for killer cell immunoglobulin like receptor, two Ig domains and a long cytoplasmic tail 4, located at 19q13.42. It belongs to a family of killer cell immunoglobulin-like receptors (KIRs), which bind to HLA class I molecules and are involved in innate immunity. *KIR2DL4* is a membrane bound receptor of natural killer cells and reported with 53 alleles in IPD-KIR database (release: 2.7.1), of which two common alleles known as 10A and 9A have been reported with controversial expression and function (23). The allele 10A encodes the full protein with 10 adenine nucleotides located in the part of the gene that encodes the transmembrane domain and the 9A allele generated by a single adenine nucleotide deletion causes a frameshift which results in premature termination of the protein product (23). These genotypes influence cell surface expression of *KIR2DL4* (23). Instead, we identified an 11A allele with a homozygous insertion of an adenine after the 10th adenine, which corresponds to a transmembrane domain in KIR2DL4. In contrast to the 9A allele, the 11A allele led to the elongation of the protein product. The resulting protein was 377 amino acids long that is similar the protein encoded by a known isoform of *KIR2DL4* (RefSeq: NM_002255.6). Thus, this variant is not a deleterious variant, which could explain the immunodeficiency observed in the patients.

IFNGR2 Splice Site Variant

A novel splice site variant c.207-1G>A (chr21:g.34793786G>A) was identified in intron 2 of *IFNGR2* gene (RefSeq: NM_005534.3) (**Figure 2A**). *IFNGR2* gene encodes for interferon gamma receptor 2 located at 21q22.11. This variant was not reported in dbSNP, 1,000 genomes project or ExAC. MutationTaster predicted the effect of this variant as disease causing (Prediction: "D"; Score: 1). In addition, the official OMIM entry (OMIM: 147569) for the *IFNGR2* gene confirms its association with Immunodeficiency 28 (IMD28) (MIM #614889), a primary immunodeficiency disease causing a genetic predisposition to mycobacterial infections by atypical Mycobacteria and the BCG vaccine (1). Of note, family history of adverse reaction to BCG vaccination was documented for two deceased older siblings of the affected individuals. This is the first study to document a possible disease-associated mutation in the splice site of *IFNGR2*. Sanger sequencing of a region around the *IFNGR2* splice acceptor site variant (c.207-1G>A) confirmed the presence of the mutation in both affected individuals as homozygous and in the parents as heterozygous (**Figure 2B**). Thus, these mutations may underlie autosomal recessive MSMD with partial or complete deficiency. Previous studies implicating *IFNGR2* in MSMD have described mutations in exonic regions affecting intracellular, transmembrane, and extracellular domains of IFNGR2 (**Figure 2C**) (24–32).

Transcript Analysis to Study the Consequences of the Splice Acceptor Site Mutation

In order to investigate any potential molecular defect caused by the identified splice acceptor site mutation from whole exome analysis, we again collected blood samples from the patients, and parents. However, the blood samples collected this time were subsequent to bone marrow transplantation. We isolated total RNA from the samples and carried out RT-PCR analysis. The RT-PCR product was run on an agarose gel and we observed that all four samples consistently showed a single band of ∼400 bp, which corresponded to the expected target of 405 bp (**Figure 3A**).

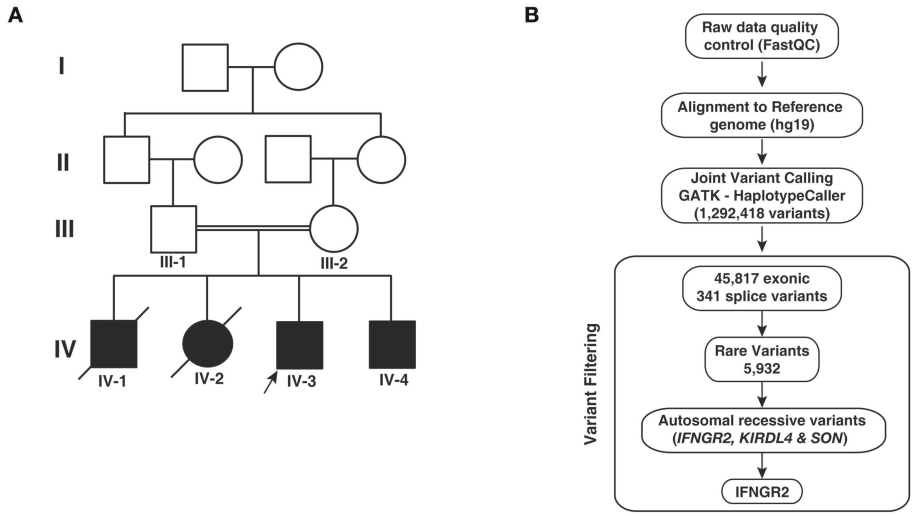

FIGURE 1 | Pedigree and analytical workflow. **(A)** Pedigree showing affected and unaffected individuals across four generations. Exome sequencing was carried out in the proband (IV-3) and his sibling (IV-4) and both parents (III-1 and III-2). **(B)** Depiction of the workflow adopted to identify potential causative variant from whole exome sequencing data.

TABLE 2 | Mutations identified in this study that are inherited as autosomal recessive mutations.

Gene symbol	Locus	Genomic change	Amino acid change	Type of mutation	Effect on protein function
IFNGR2	Chr21	g. 34793786G>A	p.(Thr70-Ser72)	Splice site	Deleterious
KIR2DL4	Chr19	g. 55324674C>CA	p.S267fs	Frameshift causing insertion	Probably benign
SON	Chr21	g.34925531C>G	p.Pro1332Ala	missense	Probably benign

We carried out Sanger sequencing of the PCR products for all four samples. Because of the splice site mutation, either exon skipping could occur or an alternate cryptic splice acceptor could be created either in intronic or exonic region of the gene. Analysis of the Sanger sequencing results consistently showed two peaks per nucleotide after the splice junction in both affected parents and patients showing the expression of both the mutant and normal forms of *IFNGR2* gene owing to the fact that the blood samples were collected after bone marrow transplantation for this analysis (**Figure 3B**). Subtracting the peaks that correspond to the normal transcript, we reconstructed the mutant *IFNGR2* transcript sequence based on the alternate peaks observed in the Sanger sequencing data (**Figure 3B**). Further, we used BLAT tool and visualized the results in a browser to verify the location of the reconstructed sequence of the alternate transcript and found that it was located after 9 nucleotides in the exon 3 downstream of the splice acceptor site mutation. Thus, the normal splice acceptor site is destroyed and an alternate cryptic splice acceptor site was created within exon 3.

Since the transcript analysis of *IFNGR2* gene in patients' blood samples showed expression of both wild type and mutant alleles (**Figure 3B**), we wanted to check the expression of wild type *IFNGR2* expression in patients fibroblast cells where no mixing with donor cells could occur. For this, we obtained skin biopsy samples from both the patients and cultured fibroblasts using standard procedures. We first isolated DNA from these cultured fibroblast cells of both the patients and amplified them using the same primers used for blood DNA amplification and carried out Sanger sequencing. We reconfirmed the homozygous splice site mutation in both the patients. In order to check the transcript level expression of *IFNGR2*, we also isolated RNA from the cultured fibroblasts and generated cDNA for amplification using the same set of primers used for transcript analysis of blood samples. Sanger sequencing showed expression of only the mutant allele confirming the 9 nucleotides deletion (**Figure 3C**).

This novel splice acceptor site mutation causes deletion of three amino acids p.(Thr70-Ser72) in *IFNGR2* (RefSeq: NP_005525.2). This deletion occurs in the FN3 domain and in Tissue_fac domain which are in the extracellular region of the receptor. The region spanning amino acids 70–73 belongs to one of the variable loop regions in the protein structure that is assumed to control the binding specificity of *IFNGR2* (33). PROVEAN predicted the effect of this deletion mutation as deleterious (Score: −5.935).

Role of *IFNGR2* in MSMD

IFNGR2 encodes for the interferon gamma receptor 2 located at 21q22.11. Human interferon-gamma receptor is a heterodimer of *IFNGR1* and *IFNGR2* (34). Both *IFNGR1* and *IFNGR2* are synthesized in endoplasmic reticulum and are transported to the golgi apparatus (35). *IFNGR2* gene is composed of 7 exons which span ∼33 kb of genomic DNA. Exon 1 and exon 2 encode a signal

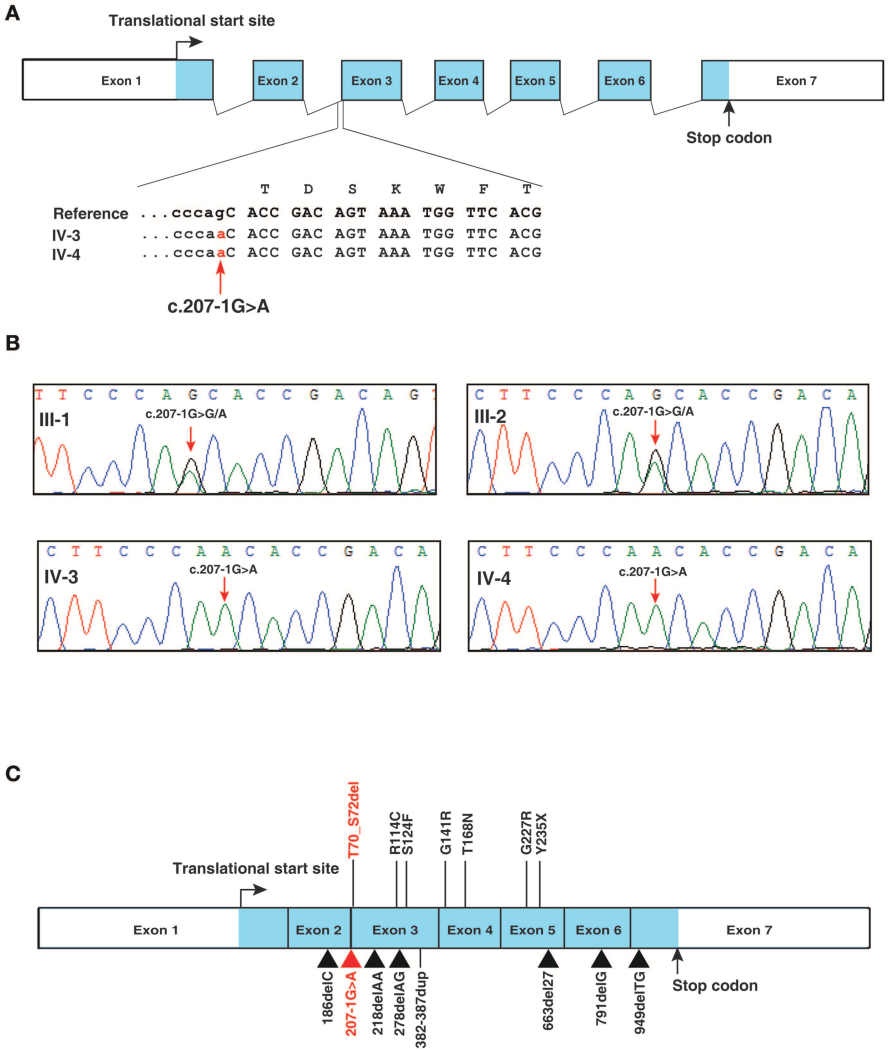

FIGURE 2 | *IFNGR2* splice site mutation. **(A)** Depiction of *IFNGR2* splice acceptor site mutation in intron 2 of *IFNGR2* gene. The changes shown are homozygous in the affected siblings **(B)** Illustration of segregation of the splice site mutation identified in *IFNGR2* gene (this study) in the affected siblings and unaffected consanguineous parents using Sanger sequencing. **(C)** Depiction of currently known mutations and the novel splice site mutation (this study) in the exon-intron architecture of *IFNGR2* gene. The splice acceptor site mutation identified in this study is marked in red and the previously reported mutations in *IFNGR2* gene are marked in black.

peptide while exons 2, 3, 4, and 5 encode for the extracellular domain. Exon 6 encodes a part of extracellular domain and the transmembrane domain while exon 7 encodes the intracellular domain and 3′UTR.

More than 27 patients have been reported thus far with several different etiologies described including (i) autosomal recessive or dominant inheritance, (ii) abolished or maintained expression of *IFNGR2*, (iii) complete or partial *IFNGR2* deficiency with or without cell surface expression, (iv) expression of non-functional *IFNGR2* on the cell surface, (v) creation of new glycosylation site that causes misfolding of the protein that abolishes the cellular responses, among many other etiologies (36).

In the first ever reported case of *IFNGR2* deficiency resulting in MSMD (24), the case history documented persistent cough and subsequent lymphadenopathy, hepatosplenomegaly, and fevers. A similar clinical presentation was observed in the proband in this study. Progressive weight loss and lymph node biopsy positive for histiocytosis were additional similarities observed between this case and confirmed MSMD cases with either complete or partial *IFNGR2* deficiency resulting from mutations in extracellular domain of the *IFNGR2* gene (24, 25) (**Figure 2C**).

Of note, while no appreciable clinical response to subcutaneous IFNγ treatment was observed in the case of complete *IFNGR2* deficiency, symptoms owing to partial *IFNGR2* deficiency could be significantly alleviated with IFNγ administration (27, 29). It is interesting to note that there are two reports of MSMD resulting from complete *IFNGR2* deficiency-causing mutations that could still be rescued with

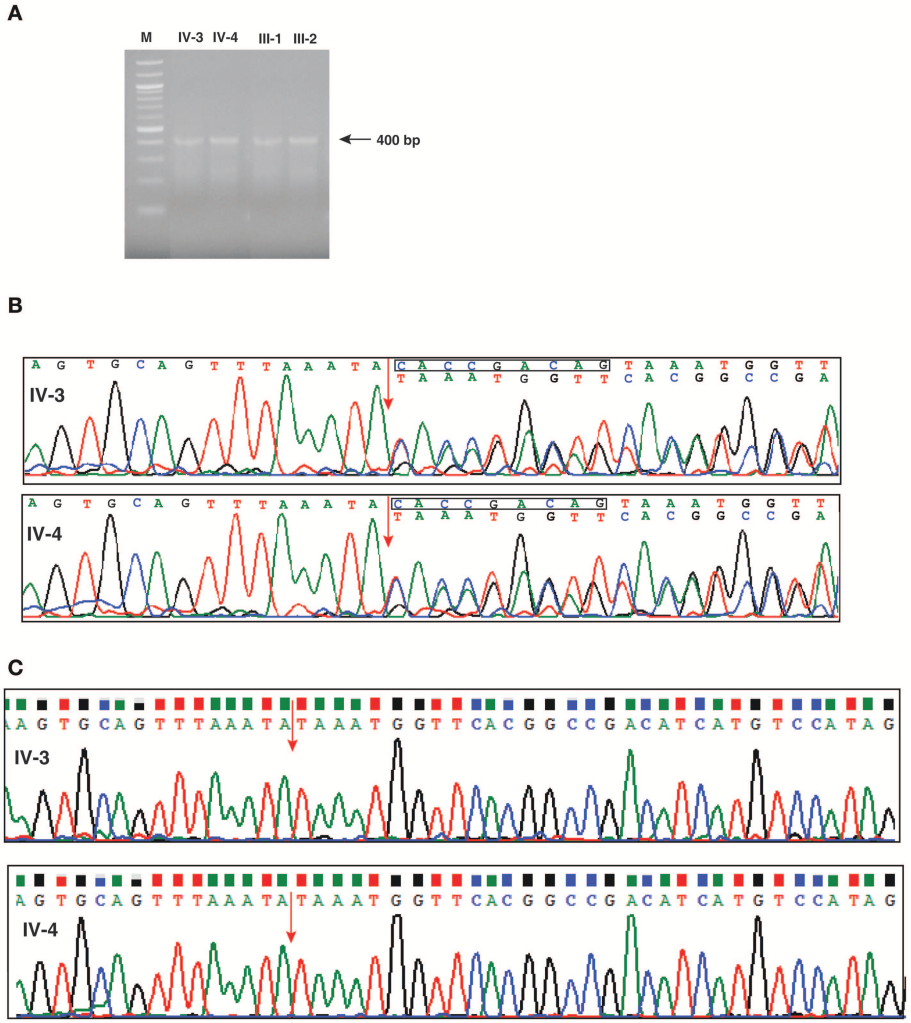

FIGURE 3 | Investigation of effect of the splice defect using reverse transcription PCR and Sanger sequencing. **(A)** Agarose gel image of the RT-PCR products targeted for a region spanning the splice acceptor site mutation of *IFNGR2* in patients and unaffected parents. The gel image shows a single band around 400 bp consistently across the patients and unaffected parents. **(B)** Depiction of the effect of splice site mutation using chromatograms obtained from the Sanger sequencing. The splice junction between exon 2 and exon 3 is shown by an red arrow. The chromatogram shows double peaks after splice junction depicting the existence of both normal and mutant *IFNGR2* transcript. The nucleotide sequences of the normal (top) and mutant transcripts (bottom) are shown. The deleted nucleotides in mutant transcripts are shown in a rectangle in the normal transcript. **(C)** Sanger sequencing results of *IFNGR2* gene isolated from patients skin fibroblast cells. The red arrow indicates the splice site of exon 2 and 3 and the sequencing data reveals deletion of nine nucleotides. Two panels represents the sequencing data of patients IV-3 and IV-4.

IFNγ administration (27, 28). In both cases, the mutations resulted in surface expression of non-functional *IFNGR2* with abnormal N-glycosylation sites. The clinical consequences in patients with complete deficiency of *IFNGR2* are often severe or fatal.

The homozygous form of *IFNGR2* splice acceptor site defect that we have observed is likely to be pathogenic leading to a potential loss of function of *IFNGR2* as suggested by the death of two siblings in the family. Given that the transplant was successful and the patients are doing well at the age of seven and 5 years respectively, it seems that a combination of thorough clinical history taking, appropriate diagnosis and the use of next generation sequencing for confirming the molecular aberration can help in choosing the most appropriate therapeutic option in affected individuals.

CONCLUSIONS

Our study underscores the importance of employing NGS-based platforms for a global exploration of primary immunodeficiencies to identify gene mutations in a clinically relevant time frame. Such studies will also aid in a more thorough understanding of the genetics of immune disorders and possibly lead to additional screening in newborns in the future. The splice acceptor site mutation identified in this study that leads to deletion of three amino acids require additional

functional studies to understand the functional implications of this mutation in immune related functions.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ICH-GCP, Indian Council of Medical Research guidelines Revised Schedule Y Guidelines of Indian Drugs and Cosmetics Rules 1945, Narayana Health Medical Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Narayana Health Medical Ethics Committee.

AUTHOR CONTRIBUTIONS

AP, SB, and MM conceptualized and designed the study. BM and PR wrote the initial draft of the manuscript. AB wrote the sections of manuscript, sample collection, processing, and performed the experiments. BM analyzed the data. SB and MM diagnosed the patients and performed the immunological assays. AD assisted in the immunological assays. PG cultured the fibroblast cells. RR helped AB for performing experiments. KR participated in the initial design of the project. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

1. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. *Semin Immunol.* (2014) 26:454–70. doi: 10.1016/j.smim.2014.09.008
2. Boisson-Dupuis S, Bustamante J, El-Baghdadi J, Camcioglu Y, Parvaneh N, El Azbaoui S, et al. Inherited and acquired immunodeficiencies underlying tuberculosis in childhood. *Immunol Rev.* (2015) 264:103–20. doi: 10.1111/imr.12272
3. Luangwedchakarn V, Jirapongsaranuruk O, NiemeLa JE, Thepthai C, Chokephaibulkit K, Sukpanichnant S, et al. A novel mutation of the IL12RB1 gene in a child with nocardiosis, recurrent salmonellosis and neurofibromatosis type I: first case report from Thailand. *Asian Pac J Allergy Immunol.* (2009) 27:161–5.
4. Aytekin C, Dogu F, Tuygun N, Tanir G, Guloglu D, Boisson-Dupuis S, et al. Bacille calmette-guerin lymphadenitis and recurrent oral candidiasis in an infant with a new mutation leading to interleukin-12 receptor beta-1 deficiency. *J Investig Allergol Clin Immunol.* (2011) 21:401–4.
5. Dorman SE, Picard C, Lammas D, Heyne K, van Dissel JT, Baretto R, et al. Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies. *Lancet.* (2004) 364:2113–21. doi: 10.1016/S0140-6736(04)17552-1
6. Sologuren I, Boisson-Dupuis S, Pestano J, Vincent QB, Fernandez-Perez L, Chapgier A, et al. Partial recessive IFN-γR1 deficiency: genetic, immunological and clinical features of 14 patients from 11 kindreds. *Hum Mol Genet.* (2011) 20:1509–23. doi: 10.1093/hmg/ddr029
7. Lee WI, Huang JL, Wu TS, Lee MH, Chen IJ, Yu KH, et al. Patients with inhibitory and neutralizing auto-antibodies to interferon-gamma resemble the sporadic adult-onset phenotype of mendelian susceptibility to mycobacterial disease (MSMD) lacking bacille calmette-guerin (BCG)-induced diseases. *Immunobiology.* (2013) 218:762–71. doi: 10.1016/j.imbio.2012.08.281
8. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. *J Clin Immunol.* (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8
9. Mimouni J. [Our experiences in three years of BCG vaccination at the center of the O.P.H.S. at Constantine; study of observed cases (25 cases of complications from BCG vaccination)]. *Alger Medicale.* (1951) 55:1138–47.
10. Jouanguy E, Altare F, Lamhamedi S, Revy P, Emile JF, Newport M, et al. Interferon-gamma-receptor deficiency in an infant with fatal bacille calmette-guerin infection. *N Engl J Med.* (1996) 335:1956–61. doi: 10.1056/NEJM199612263352604
11. Oleaga-Quintas C, Deswarte C, Moncada-Velez M, Metin A, Krishna Rao I, Kanik-Yuksek S, et al. A purely quantitative form of partial recessive IFN-gammaR2 deficiency caused by mutations of the initiation or second codon. *Hum Mol Genet.* (2018) 27:3919–35. doi: 10.1093/hmg/ddy275
12. Rosain J, Kong XF, Martinez-Barricarte R, Oleaga-Quintas C, Ramirez-Alejo N, Markle J, et al. Mendelian susceptibility to mycobacterial disease: 2014-2018 update. *Immunol Cell Biol.* (2018) 97:360–7. doi: 10.1111/imcb.12210
13. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, et al. International union of immunological societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *J Clin Immunol.* (2018) 38:96–128. doi: 10.1007/s10875-017-0464-9
14. Filipe-Santos O, Bustamante J, Chapgier A, Vogt G, de Beaucoudrey L, Feinberg J, et al. Inborn errors of IL-12/23- and IFN-γ-mediated immunity: molecular, cellular, and clinical features. *Semin Immunol.* (2006) 18:347–61. doi: 10.1016/j.smim.2006.07.010
15. de Vries E. Patient-centred screening for primary immunodeficiency: a multi-stage diagnostic protocol designed for non-immunologists. *Clin Exp Immunol.* (2006) 145:204–14. doi: 10.1111/j.1365-2249.2006.03138.x
16. Fieschi C, Bosticardo M, de Beaucoudrey L, Boisson-Dupuis S, Feinberg J, Santos OF, et al. A novel form of complete IL-12/IL-23 receptor beta1 deficiency with cell surface-expressed nonfunctional receptors. *Blood.* (2004) 104:2095–101. doi: 10.1182/blood-2004-02-0584
17. Cossu F. Genetics of SCID. *Ital J Pediatr.* (2010) 36:76. doi: 10.1186/1824-7288-36-76
18. Cottle LE. Mendelian susceptibility to mycobacterial disease. *Clin Genet.* (2011) 79:17–22. doi: 10.1111/j.1399-0004.2010.01510.x

19. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-γ-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol.* (1999) 163:2267–75.
20. Sawitzki B, Schlickeiser S, Reinke P, Volk HD. Monitoring tolerance and rejection in organ transplant recipients. *Biomarkers.* (2011) 16(Suppl 1):S42–50. doi: 10.3109/1354750X.2011.578754
21. Muthusamy B, Selvan LDN, Nguyen TT, Manoj J, Stawiski EW, Jaiswal BS, et al. Next-generation sequencing reveals novel mutations in X-linked intellectual disability. *OMICS.* (2017) 21:295–303. doi: 10.1089/omi.2017.0009
22. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics.* (2015) 31:2745–7. doi: 10.1093/bioinformatics/btv195
23. Goodridge JP, Lathbury LJ, John E, Charles AK, Christiansen FT, Witt CS. The genotype of the NK cell receptor, KIR2DL4, influences INFgamma secretion by decidual natural killer cells. *Mol Hum Reprod.* (2009) 15:489–97. doi: 10.1093/molehr/gap039
24. Dorman SE, Holland SM. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J Clin Invest.* (1998) 101:2364–9. doi: 10.1172/JCI2901
25. Doffinger R, Jouanguy E, Dupuis S, Fondaneche MC, Stephan JL, Emile JF, et al. Partial interferon-gamma receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and *Mycobacterium abscessus* infection. *J Infect Dis.* (2000) 181:379–84. doi: 10.1086/315197
26. Rosenzweig SD, Dorman SE, Uzel G, Shaw S, Scurlock A, Brown MR, et al. A novel mutation in IFN-γ receptor 2 with dominant negative activity: biological consequences of homozygous and heterozygous states. *J Immunol.* (2004) 173:4000–8. doi: 10.4049/jimmunol.173.6.4000
27. Vogt G, Chapgier A, Yang K, Chuzhanova N, Feinberg J, Fieschi C, et al. Gains of glycosylation comprise an unexpectedly large group of pathogenic mutations. *Nat Genet.* (2005) 37:692–700. doi: 10.1038/ng1581
28. Vogt G, Bustamante J, Chapgier A, Feinberg J, Boisson Dupuis S, Picard C, et al. Complementation of a pathogenic *IFNGR2* misfolding mutation with modifiers of N-glycosylation. *J Exp Med.* (2008) 205:1729–37. doi: 10.1084/jem.20071987
29. Toyoda H, Ido M, Nakanishi K, Nakano T, Kamiya H, Matsumine A, et al. Multiple cutaneous squamous cell carcinomas in a patient with interferon gamma receptor 2 (IFN gamma R2) deficiency. *J Med Genet.* (2010) 47:631–4. doi: 10.1136/jmg.2009.072108
30. Kilic SS, van Wengen A, de Paus RA, Celebi S, Meziane B, Hafizoglu D, et al. Severe disseminated mycobacterial infection in a boy with a novel mutation leading to IFN-γR2 deficiency. *J Infect.* (2012) 65:568–72. doi: 10.1016/j.jinf.2012.08.008
31. Kong XF, Vogt G, Itan Y, Macura-Biegun A, Szaflarska A, Kowalczyk D, et al. Haploinsufficiency at the human *IFNGR2* locus contributes to mycobacterial disease. *Hum Mol Genet.* (2013) 22:769–81. doi: 10.1093/hmg/dds484
32. Moncada-Velez M, Martinez-Barricarte R, Bogunovic D, Kong XF, Blancas-Galicia L, Tirpan C, et al. Partial IFN-γR2 deficiency is due to protein misfolding and can be rescued by inhibitors of glycosylation. *Blood.* (2013) 122:2390–401. doi: 10.1182/blood-2013-01-480814
33. Mikulecky P, Zahradnik J, Kolenko P, Cerny J, Charnavets T, Kolarova L, et al. Crystal structure of human interferon-gamma receptor 2 reveals the structural basis for receptor specificity. *Acta Crystallogr D Struct Biol.* (2016) 72(Pt 9):1017–25. doi: 10.1107/S2059798316012237
34. Bhat MY, Solanki HS, Advani J, Khan AA, Keshava Prasad TS, Gowda H, et al. Comprehensive network map of interferon gamma signaling. *J Cell Commun Signal.* (2018) 12:745–51. doi: 10.1007/s12079-018-0486-y
35. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol.* (1993) 11:571–611. doi: 10.1146/annurev.iy.11.040193.003035
36. Oleaga-Quintas C, Deswarte C, Moncada-Velez M, Metin A, Krishna Rao I, Kanik-Yuksek S, et al. A purely quantitative form of partial recessive IFN-gammaR2 deficiency caused by mutations of the initiation or second codon. *Hum Mol Genet.* (2019) 28:524. doi: 10.1093/hmg/ddy357

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frontiers in Immunology

CASE REPORT
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Immunological Features of Neuroblastoma Amplified Sequence Deficiency: Report of the First Case Identified Through Newborn Screening for Primary Immunodeficiency and Review of the Literature

Silvia Ricci[1†], Lorenzo Lodi[1†], Daniele Serranti[2], Marco Moroni[3], Gilda Belli[3], Giorgia Mancano[4], Andrea La Barbera[4], Giulia Forzano[4], Giusi Mangone[1], Giuseppe Indolfi[2] and Chiara Azzari[1]*

[1] Section of Pediatrics, Division of Immunology, Department of Health Sciences, Meyer Children's University Hospital, Florence, Italy, [2] Pediatric and Liver Unit, Meyer Children's University Hospital, Florence, Italy, [3] Neonatal Intensive Care Unit, Department of Pediatrics, Meyer Children's University Hospital, Florence, Italy, [4] Medical Genetics Unit, Meyer Children's University Hospital, Florence, Italy

This is the first case of NBAS disease detected by NBS for primary immunodeficiency. NBS with KRECs is revealing unknown potentialities detecting conditions that benefit from early recognition like NBAS deficiency. Immune phenotyping should be mandatory in patients with NBAS deficiency since they can exhibit severe immunodeficiency with hypogammaglobulinemia as the most frequent finding. Fever during infections is a known trigger of acute liver failure in this syndrome, so immune dysfunction, should never go unnoticed in NBAS deficiency in order to start adequate therapy and prophylaxis.

Keywords: KREC, newborn screening, immunodeficiency, NBAS, neuroblastoma amplified sequence deficiency, SOPH, ILFS2

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Antonio Condino-Neto, University of São Paulo, Brazil

Reviewed by:
Janet Chou, Boston Children's Hospital and Harvard Medical School, United States
Elham Hossny, Ain Shams University, Egypt
Mirjam van der Burg, Leiden University Medical Center, Netherlands
Reza Yazdani, Tehran University of Medical Sciences, Iran

***Correspondence:**
Silvia Ricci
silvia.ricci@meyer.it

[†] These authors have contributed equally to this work

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BACKGROUND

Neuroblastoma amplified sequence (NBAS) deficiency is a very rare disease characterized by an extremely broad spectrum of phenotypes (1). Clinical features range from isolated recurrent episodic liver failure, precipitated by intercurrent febrile illness, to multisystemic syndrome, which includes short stature, skeletal osteopenia and dysplasia, optic atrophy, immunological abnormalities, and autoimmune disorders (2). NBAS is a highly conserved gene encoding for a component of the syntaxin-18 complex, a component of an endoplasmic reticulum (ER) tethering complex involved in the Golgi–ER retrograde transport of vesicles (3). Thermal susceptibility of the syntaxin-18 complex is the basis of fever dependency of liver failure episodes and could be involved in the possible pathogenic mechanism of NBAS mutations.

In this paper, we report the first case of NBAS deficiency identified in the context of a newborn screening (NBS) program for primary immunodeficiencies (PIDs) with κ-deleting recombination excision circles (KRECs) quantification on dried blood spot. We also focus on the immunological spectrum of the disease with a review of immunological cases in the literature.

CASE PRESENTATION

The patient is a male newborn of Pakistani origin, son of consanguineous parents (**Figure S1**—Supplementary Material section), born by scheduled cesarean section at 36 weeks of gestational age, with severe intrauterine growth restriction (−3DS) and blood flow abnormalities at a prenatal Doppler ultrasonography.

The patient came to our attention due to the complete absence of KRECs and normal TRECs on DBS (dried blood spot) while hospitalized for low weight at birth (1,520 g), intolerance for enteral feeding, hepatosplenomegaly, slightly elevated liver transaminase, head and face eczematous dermatitis, and persistent Rotavirus enteritis. During the 1st month, he also presented *Klebsiella pneumoniae* urinary tract infection and methicillin-resistant *Staphylococcus aureus* sepsis. Peculiar phenotypic features including triangular face, proptosis, flat philtrum, mild retrognatia, hirsutism, loose and slightly wrinkled skin, and apparent reduction of subcutaneous fat were noticed at birth. Complete blood count showed lymphocytopenia out of infectious episodes, marked hypereosinophilia, and a low platelet count with normal mean volume. Serum immunoglobulin G (IgG) were markedly decreased, IgA and IgM were undetectable, and levels of IgE were slightly augmented (**Table 1**). Extended phenotyping of the immune system was carried out on peripheral blood (**Table 1**, **Figure 1**) and confirmed on whole blood the normal expression of TRECs and the complete absence of KRECs, complete absence of CD19+ cells, low count of CD8+ lymphocytes, and reduced natural killer (NK) levels (**Table 1**, **Figure 1**). Classical and leaky forms of severe combined immunodeficiency were excluded by normal proliferation response of T cells to mitogens. Maternal engraftment of T lymphocytes was excluded by the normal representation of naïve T cells and by the different HLA-I typing and karyotypes of mother and son. The T cell receptor (TCR) repertoire expressed normal variability. Flow cytometric analysis showed normal expression of BTK (Bruton tyrosine kinase) protein on monocytes (**Figure S2**—Supplementary Material section) and normal expression of wasp protein on lymphocytes and monocytes. Regulatory T cells were normally represented among T CD4+ lymphocytes (**Table 1**). A colonoscopy was carried out for persistent diarrhea and reduced tolerance to enteral feeding. The histological examination of mucosal intestinal biopsies showed an absence of plasma cells and reduced representation of T lymphocytes, suggesting immunodeficiency but not autoimmune enteropathy. Molecular analysis for genome detection of adenovirus, rotavirus, EBV, CMV, and enterovirus were carried out on intestinal biopsies and no viral copies were detected. As a syndromic picture was suspected, clinical exome was performed by Next Generation Sequencing and identified a homozygous variant in NBAS (NM_015909): c. [1948C>T], p.Pro650Ser, inherited from both parents (**Figure S1**—Supplementary material section). This variant has not been described in the literature and is reported as rs558233705 with a low allele frequency in the Asian population in principal exome and genome databases (https://www.ncbi.nlm.nih.gov/snp/rs558233705#frequency_tab. Last visit on 05 February 2019).

To investigate the NBAS mutation-based disease features associated, total body X-ray was performed and revealed slightly bilateral brachydactilia of the 5th finger.

Immunoglobulin substitutive therapy and antimicrobial prophylaxis were promptly started during the 1st days of life. At the age of 3 months, due to the persistence of clinical manifestations with severe growth restriction and severe eczematous dermatitis, corticosteroid therapy was introduced with rapid improvement of gastrointestinal and cutaneous manifestations and the almost immediate reduction of peripheral hypereosinophilia. Steroid tapering was attempted unsuccessfully and caused re-exacerbation of gastrointestinal symptoms which, at 7 months of age, still constitute the most consistent clinical finding, together with severe growth restriction.

MATERIALS AND METHODS

Written informed consent has been obtained from the legal representatives of the patient for the publication of this case report.

NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY WITH KRECS

The forward, reverse primers and probes for KRECs were designed in our laboratory using Primer Express software version 3.0 (Applied Biosystems). Primer specificity was assessed by BLAST search (http://www.ncbi.nlm.nih.gov/blast/), which confirmed their uniqueness. TREC and KREC levels were normalized per microliter of blood, assuming that the sample contains approximately 3 μl of whole blood. Calibration curves were generated by means of 10-fold serial dilution of plasmids that contain TREC, KREC, and β-actin sequences. Diagnostic cutoff scores were established at 25 TRECs/μl and 10 KRECs/μl, according to the values reported in literature (4).

WHOLE-EXOME SEQUENCING

Exomes of the patient and his parents were captured from the genomic DNA using SeqCap EZ MedExome (Roche NimbleGen) and sequencing was carried out using the NextSeq Illumina platform. The alignment of the reads (BWA), the call of the variants (GATK), the annotation (Annovar), and the

Abbreviations: NBAS, neuroblastoma amplified sequence; TRECs, T-cell receptor excision circles; KRECs, κ-deleting recombination excision circles; SOPH, short stature with optic atrophy and Pelger–Huët anomaly syndrome; ILFS2, type 2 infantile hepatic impairment.

TABLE 1 | Patient's immune phenotyping at different ages.

Age	1 month and 20 days	2-months	3-months	4-months	5-months	6-months	7-months
Lymphocyte count (cells/mcL) (% of WB)							
Lymphocyte subtypes (cells/mcL) (% of lymphocytes)	2,813 (16,1%)	3,112 (18.5%)	714 (10%)	1,550 (21%)	1,835 (20%)	2,081 (15.1%)	638 (8.8%)
CD19+	4 (0%)	7 (0%)	3 (0%)	0 (0%)	0 (0%)	n.a.	n.a.
CD3+	2,348 (88%)	4,428 (95%)	1,963 (97%)	1,860 (95%)	1,575 (95%)	n.a.	n.a.
CD3+CD4+	2,127 (80%)	3,714 (79%)	1,396 (69%)	1,396 (72%)	1,370 (83%)	n.a.	n.a.
CD3+CD8+	189 (7%)	566 (12%)	389 (19%)	401 (21%)	186 (11%)	n.a.	n.a.
CD3−CD16+CD56+	261 (10%)	193 (4%)	43 (2%)	72 (4%)	60 (4%)	n.a.	n.a.
CD4+/CD8+ ratio	11.4	6.6	3.6	3.4	7.5	n.a.	n.a.
Specific lymphocyte counts (% of CD4+)							
CD45RA+	78%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
CD45RO+	22%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
$CD25+CD127^{Low}$	n.a.	6.7 (n.r. 4–16)	n.a.	n.a.	n.a.	n.a.	n.a.
Mitogen stimulation							
PHA	85 (n.v. > 80)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IL-2	83 (n.v. > 80)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Serum immunoglobulins							
IgG (mg/dl)	325	213	n.a.	182	252	307	539
IgA (mg/dl)	<7.83	<7.83	n.a.	<7.83	<7.83	<7.83	8.2
IgM (mg/dl)	24	34	n.a.	<52.5	35	32	18
IgE (kU/L)	108	11	n.a.	n.a.	n.a.	n.a.	n.a.

n.a., not available; n.v., normal values.

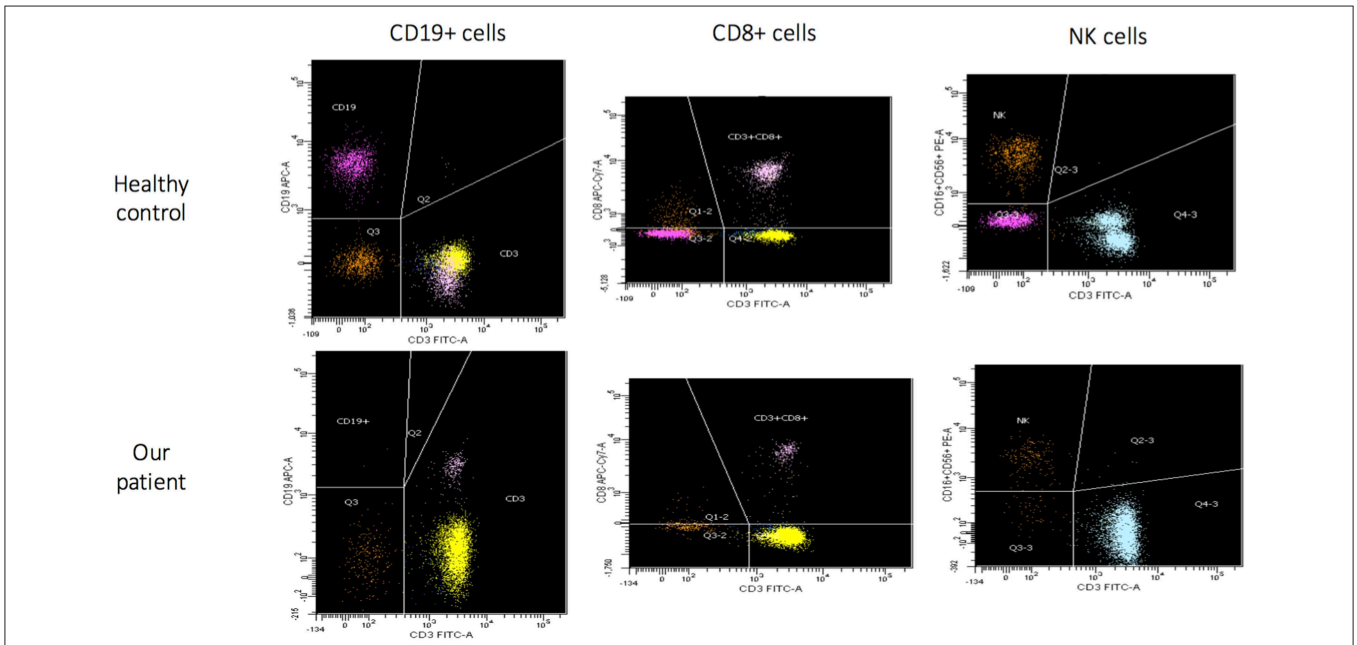

FIGURE 1 | Flow cytometric assessment of lymphocyte subsets in healthy control and in our patient (age matched) that show for our patient absence of B cells (CD19+), low CD8+ and NK cells.

prioritization of the variants were performed with strategies developed in-house according to the guidelines of the American College of Medical Genetics and Genomics (ACMG). The data obtained were filtered according to the patient's clinical phenotype. A homozygous variant in NBAS (NM_015909): c.1948C > T, inherited from both parents, was identified. The 1948C > T variant leads to the substitution of hydrophobic proline 650 with polar serine (p.Pro650Ser).

DISCUSSION

This is the first reported case of the NBAS mutation-based disease detected by NBS for PIDs with KRECs quantification on DBS. Bi-allelic mutations in the NBAS gene cause a wide range of phenotypes, including hepatic, skeletal, ocular, and immunologic abnormalities. T-cell receptor excision circles (TRECs) and KRECs are used worldwide for detection of T or B cell lymphopenia in newborns. As previously described (5), Tuscany is the only region in Italy to have implemented an NBS for PIDs with TREC and KREC quantification on DBS through multiplex real-time PCR (polymerase chain reaction). By January 2019, 38,300 children had been screened. The implementation and use of NBS for PIDs with TREC and KREC in a large-scale population are revealing otherwise unknown potentialities of the method. Not only does it allow the identification of known cellular and humoral PIDs, but it also permits the detection of clinically severe congenital conditions that benefit from early recognition and that are variably associated with immunodeficiency, such as NBAS deficiency. NBAS mutation-based disease has been associated with two main clinical phenotypes: the infantile liver failure syndrome 2 (ILFS2) and short stature with optic atrophy and Pelger–Huët anomaly syndrome (SOPH) (2, 6). The liver phenotype in NBAS deficiency is most typically a recurrent acute liver failure, triggered by febrile infections. The molecular pathogenesis by which an NBAS defect contributes to fever-dependent liver disease is not fully understood. The NBAS protein is thought to be involved in Golgi-to-ER retrograde vesicular trafficking and to control non-sense-mediated mRNA decay (7). A knockdown of NBAS in HeLa cells led to a defective protein glycosylation (3). Staufner et al. described how fibroblasts of patients with bi-allelic NBAS mutations showed an increased susceptibility to high temperatures at the protein and functional levels, causing an impaired transport between Golgi-to-ER retrograde vesicular trafficking. They suggested that raised temperatures themselves may be the starting point of ER stress-induced liver

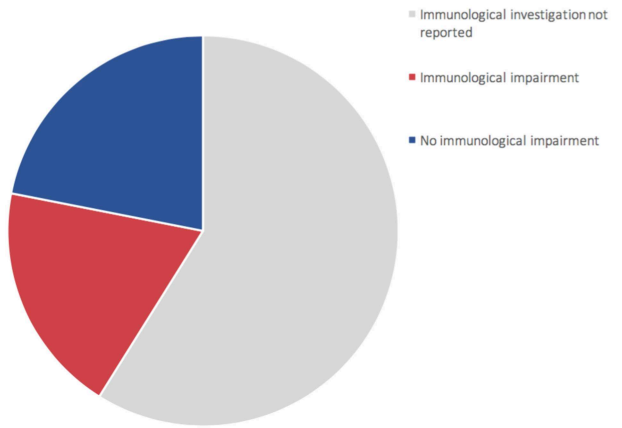

FIGURE 2 | Representation of 73 patients reported in literature (1, 6, 8–22). 60% of patients (gray area) did not undergo complete immune characterization. 44% of patients who underwent immune characterization presented an immunological impairment (red area).

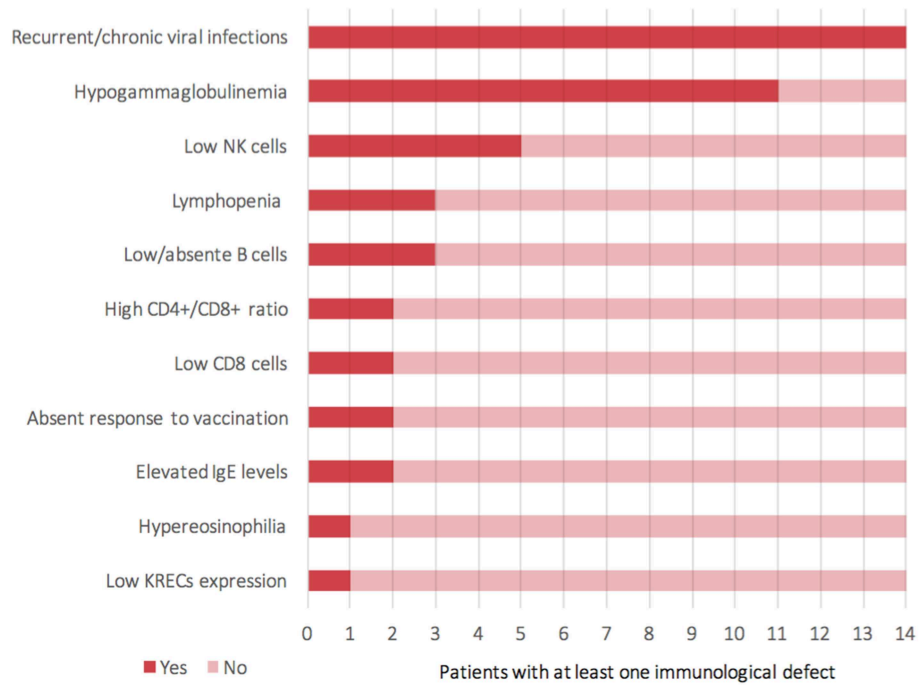

FIGURE 3 | Representation of clinical and laboratory immunological findings (Y-axis) reported among 14 patients (X-axis) who presented a known immunological impairment (including the current case).

cell apoptosis that lead to fever-dependent ALF (acute liver failure) (1).

For the time being, our patient has not presented episodes of ALF, but persistent elevations of transaminase have been detected (**Figure S3**—Supplementary material section) with normal cholestasis values and coagulation function. The last abdominal ultrasound evaluation (performed during his 7th month) revealed liver of fairly increased size associated with preserved biliary tract features and liver echostructure. He presents facial dimorphisms and slight bilateral brachydactyly of the 5th finger, but no optical atrophy and no Pelger–Huët anomalies have been identified. Due to his young age, it is still difficult to determine whether the clinical phenotype tends toward ILFS2 or to SOPH. As in two previously described cases (8), the immunological impairment, associated with the gastrointestinal dysfunction, seems to be predominant in the global clinical picture of our patient. The literature reviewed revealed that, in more than half of the patients with NBAS deficiency, immune characterization is completely unknown (**Figure 2**) or partial (1, 6, 8–22). In particular, a complete absence of B cells has never been described in NBAS deficiency, but recurrent/chronic viral infections, severe hypogammaglobinemia, and a reduced number of B cells are the most frequently described immune anomalies in patients with NBAS deficiency (**Figure 3**) (1, 6, 8–22). Moreover, a reduced number of normally functional NK cells, altered CD4+/CD8+ ratio with reduced CD8+ numbers and hyper-IgE, have been previously described and are present in our patient (**Figure 3**). Our patient seems to possess the whole range of immune alterations hitherto described in previous cases, thus exhibiting the most severe immune phenotype ever reported in NBAS deficiency.

Immunodeficiency, associated with the NBAS phenotype, contributes to recurrent infections and abnormal liver enzymes with fever. Furthermore, since fever is a known trigger of acute liver failure, immune dysfunction, even if mild, should never go unnoticed in NBAS deficiency. In particular, hypogammaglobinemia is the most frequent immunological feature associated with NBAS deficiency (**Figure 3**) (1, 6, 8–22); thus, these patients may benefit from replacement therapy with immunoglobulin in order to reduce the rate of infections and the consequent risk of acute liver failure. We suggest that extended immune phenotyping should be mandatory in all these patients in order to collect and exchange data regarding a disease whose characterization is still being unfolding. In fact, basing on previously reported NBAS cases in literature, it is not possible to define a clear genotype–phenotype correlation regarding immunological involvement in this syndrome (**Figure S4**) (1, 6, 8–22). The phenotype classification for PID outlined by the International Union of Immunological Societies (IUIS) already includes NBAS deficiency among inborn errors of immunity (23). If the high frequency of hypogammaglobinemia in NBAS deficiency is confirmed (**Figure 2**), the authors feel that inclusion among prevalent antibody deficiencies could be of even greater support for the clinicians facing such a challenging diagnostic process. The potential mechanism of NBAS mutations affecting the immune system is still unknown. Further in-depth functional studies on Golgi-to-ER retrograde vesicular trafficking in immune cells of affected patients are needed to establish the genotype–phenotype correlations and to reveal the pathogenesis of the wide immunological phenotypic spectrum of the NBAS disease.

ETHICS STATEMENT

A specific approval by the local ethical committee was not required because all analyses included in this study had been performed as part of the routine clinical activity. All data have been anonymized. Written informed consent was obtained from the parents of the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

MM and GB evaluated the patient during his NICU hospitalization. Evaluation on hepatic and immunological aspects was carried out by DS and SR, respectively. GManc and AL carried out the molecular diagnosis. GF collected the data for family pedigree. GMang performed the immunological tests. SR and LL collected the clinical and immunological data, reviewed the literature, wrote the first draft of the article, and critically revised it. GI and CA, as experts, critically revised the article and gave their final approval for submission. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01955/full#supplementary-material

REFERENCES

1. Staufner C, Haack TB, Köpke MG, Straub BK, Kölker S, Thiel C, et al. Recurrent acute liver failure due to NBAS deficiency: phenotypic spectrum, disease mechanisms, and therapeutic concepts. *J Inherit Metab Dis.* (2016) 39:3–16. doi: 10.1007/s10545-015-9896-7

2. Haack TB, Staufner C, Köpke MG, Straub BK, Kölker S, Thiel C, et al. Biallelic mutations in NBAS cause recurrent acute liver failure with onset in infancy. *Am J Hum Genet.* (2015) 97:163–9. doi: 10.1016/j.ajhg.2015.05.009

3. Aoki T, Ichimura S, Itoh A, Kuramoto M, Shinkawa T, Isobe T, et al. Identification of the neuroblastoma-amplified gene product as

a component of the syntaxin 18 complex implicated in Golgi-to-endoplasmic reticulum retrograde transport. *Mol Biol Cell.* (2009) 20:2639–49. doi: 10.1091/mbc.e08-11-1104
4. 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) 119:2552–5. doi: 10.1182/blood-2011-08-37102
5. Lodi L, Ricci S, Romano F, Ghiori F, Canessa C, Lippi F, et al. Newborn screening for PIDs using both TREC and KREC identifies late occurrence of B cells. *Pediatr Allergy Immunol.* (2017) 28:498–500. doi: 10.1111/pai.12733
6. Maksimova N, Hara K, Nikolaeva I, Chun-Feng T, Usui T, Takagi M, et al. Neuroblastoma amplified sequence gene is associated with a novel short stature syndrome characterized by optic nerve atrophy and Pelger-Huët anomaly. *J Med Genet.* (2010) 47:538–48. doi: 10.1136/jmg.2009.074815
7. Hug N, Longman D, Cáceres JF. Mechanism and regulation of the nonsense-mediated decay pathway. *Nucleic Acids Res.* (2016) 44:1483–95. doi: 10.1093/nar/gkw010
8. Segarra NG, Ballhausen D, Crawford H, Perreau M, Campos-Xavier B, van Spaendonck-Zwarts K, et al. NBAS mutations cause a multisystem disorder involving bone, connective tissue, liver, immune system, and retina. *Am J Med Genet A.* (2015) 167A:2902–12. doi: 10.1002/ajmg.a.37338
9. Nucci F, Lembo A, Farronato M, Farronato G, Nucci P, Serafino M. Oculofacial alterations in NBAS-SOPH like mutations: case report. *Eur J Ophthalmol.* (2019) 8:1120672119836335. doi: 10.1177/1120672119836335
10. Ono S, Matsuda J, Watanabe E, Akaike H, Teranishi H, Miyata I, et al. Novel neuroblastoma amplified sequence (NBAS) mutations in a Japanese boy with fever-triggered recurrent acute liver failure. *Hum Genome Var.* (2019) 6:2. doi: 10.1038/s41439-018-0035-5
11. Carli D, Giorgio E, Pantaleoni F, Bruselles A, Barresi S, Riberi E, et al. NBAS pathogenic variants: defining the associated clinical and facial phenotype and genotype–phenotype correlations. *Hum Mutat.* (2019) 40:721–8. doi: 10.1002/humu.23734
12. Li X, Cheng Q, Li N, Chang G, Ding Y, Li J, et al. SOPH syndrome with growth hormone deficiency, normal bone age, and novel compound heterozygous mutations in NBAS. *Fetal Pediatr Pathol.* (2018) 37:404–10. doi: 10.1080/15513815.2018
13. Palagano E, Zuccarini G, Prontera P, Borgatti R, Stangoni G, Elisei S, et al. Mutations in the neuroblastoma amplified sequence gene in a family affected by acrofrontofacionasal dysostosis type 1. *Bone.* (2018) 114:125–36. doi: 10.1016/j.bone.2018.06.013
14. Sunwoo Y, Kim YM, Kim EN, Oh SH, Lee BH. Severe form of neuroblastoma amplified sequence deficiency in an infant with recurrent acute liver failure. *Pediatr Int.* (2018) 60:302–4. doi: 10.1111/ped.13476
15. Wang J, Pu Z, Lu Z. Targeted next-generation sequencing reveals two novel mutations of NBAS in a patient with infantile liver failure syndrome-2. *Mol Med Rep.* (2018) 17:2245–50. doi: 10.3892/mmr.2017.8191
16. Calvo PL, Tandoi F, Haak TB, Brunati A, Pinon M, Olio DD, et al. NBAS mutations cause acute liver failure: when acetaminophen is not a culprit. *Ital J Pediatr.* (2017) 43:88. doi: 10.1186/s13052-017-0406-4
17. Li JQ, Qiu YL, Gong JY, Dou LM, Lu Y, Knisely AS, et al. Novel NBAS mutations and fever-related recurrent acute liver failure in Chinese children: a retrospective study. *BMC Gastroenterol.* (2017) 17:77. doi: 10.1186/s12876-017-0636-3
18. Regateiro FS, Belkaya S, Neves N, Ferreira S, Silvestre P, Lemos S, et al. Recurrent elevated liver transaminases and acute liver failure in two siblings with novel bi-allelic mutations of NBAS. *Eur J Med Genet.* (2017) 60:426–32. doi: 10.1016/j.ejmg.2017.05.005
19. Park JW, Lee SJ. Foveal hypoplasia in short stature with optic atrophy and Pelger–Huët anomaly syndrome with neuroblastoma-amplified sequence (NBAS) gene mutation. *J AAPOS.* (2017) doi: 10.1016/j.jaapos.2016.09.030
20. Kortüm F, Marquardt I, Alawi M, Korenke GC, Spranger S, Meinecke P, et al. Acute liver failure meets SOPH syndrome: a case report on an intermediate phenotype. *Pediatrics.* (2017) 139:e20160550. doi: 10.1542/peds.2016-0550
21. Balasubramanian M, Hurst J, Brown S, Bishop NJ, Arundel P, DeVile C, et al. Compound heterozygous variants in NBAS as a cause of atypical osteogenesis imperfecta. *Bone.* (2017) 94:65–74. doi: 10.1016/j.bone.2016.10.023
22. Capo-Chichi JM, Mehawej C, Delague V, Caillaud C, Khneisser I, Hamdan FF, et al. Neuroblastoma amplified sequence (NBAS) mutation in recurrent acute liver failure: confirmatory report in a sibship with very early onset, osteoporosis and developmental delay. *Eur J Med Genet.* (2015) 58:637–41. doi: 10.1016/j.ejmg.2015.11.005
23. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. *J Clin Immunol.* (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8

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frontiers in Immunology

ORIGINAL RESEARCH
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Quantification of T-Cell and B-Cell Replication History in Aging, Immunodeficiency, and Newborn Screening

*Ruud H. J. Verstegen[1,2,3], Pei M. Aui[4,5], Eliza Watson[4,5], Samuel De Jong[4], Sophinus J. W. Bartol[1], Julian J. Bosco[5,6], Paul U. Cameron[5,6], Robert G. Stirling[5,6], Esther de Vries[7,8], Jacques J. M. van Dongen[9] and Menno C. van Zelm[1,4,5,6]**

[1] Department of Immunology, Erasmus MC, University Medical Centre, Rotterdam, Netherlands, [2] Division of Rheumatology, Department of Paediatrics, The Hospital for Sick Children, Toronto, ON, Canada, [3] Division of Clinical Pharmacology and Toxicology, Department of Paediatrics, The Hospital for Sick Children, Toronto, ON, Canada, [4] Department of Immunology and Pathology, Monash University, Melbourne, VIC, Australia, [5] The Jeffrey Modell Diagnostic and Research Centre for Primary Immunodeficiencies, Melbourne, VIC, Australia, [6] Department of Allergy, Immunology and Respiratory Medicine, The Alfred Hospital, Melbourne, VIC, Australia, [7] Tranzo, Scientific Center for Care and Welfare, Tilburg University, Tilburg, Netherlands, [8] Laboratory for Medical Microbiology and Immunology, Elisabeth-TweeSteden Hospital, Tilburg, Netherlands, [9] Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, Leiden, Netherlands

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Edited by:
Antonio Condino-Neto, University of São Paulo, Brazil

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***Correspondence:**
Menno C. van Zelm
menno.vanzelm@monash.edu

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Quantification of T-cell receptor excision circles (TRECs) has impacted on human T-cell research, but interpretations on T-cell replication have been limited due to the lack of a genomic coding joint. We here overcome this limitation with multiplex TRG rearrangement quantification (detecting ∼0.98 alleles per TCRαβ+ T cell) and the HSB-2 cell line with a retrovirally introduced TREC construct. We uncovered <5 cell divisions in naive and >10 cell divisions in effector memory T-cell subsets. Furthermore, we show that TREC dilution with age in healthy adults results mainly from increased T cell replication history. This proliferation was significantly increased in patients with predominantly antibody deficiency. Finally, Guthrie cards of neonates with Down syndrome have fewer T and B cells than controls, with similar T-cell and slightly higher B-cell replication. Thus, combined analysis of TRG coding joints and TREC signal joints can be utilized to quantify *in vivo* T-cell replication, and has direct applications for research into aging, immunodeficiency, and newborn screening.

Keywords: T-cell replication, TREC, TRG, primary immunodeficiency, newborn screening, aging

INTRODUCTION

Adaptive immunity is a critical component of the vertebrate immune system and is represented cellularly by B- and T-lymphocytes. Their crucial roles are illustrated in patients with inborn errors of immunity (IEI) (1). For example, patients with severe combined immunodeficiency (SCID) primarily lack mature T cells resulting in a lethal immunodeficiency if untreated (2). Predominantly antibody deficiency (PAD) is more common (3), and infectious complications in these patients can be managed with immunoglobulin replacement and prophylactic antibiotics. Still, about 68% of patients develop non-infectious complications, including autoimmunity and malignancies, which lead to high morbidity and early mortality (4–6). Hence, there is a need for early diagnosis of both severe and milder forms of IEI, as well as reliable markers that could predict future complications.

Similar to all blood cells, B and T lymphocytes are continuously produced throughout life. Progenitor B and T cells in bone marrow and thymus, respectively, generate unique antigen receptors through genomic rearrangements of their immunoglobulin (Ig) and T-cell receptor loci. In this process, coding joints are formed on chromosomes, and signal joints on circular excision products that are stably present in the cell, but are not replicated during cell divisions (7). Newly-formed T cells carry T-cell receptor excision circles (TRECs), whereas in memory T-cell populations these are extremely diluted following cell divisions. As such, TRECS are markers for thymic output (8). Indeed, PCR-based quantitative detection of TRECs has been applied to examine the effects of novel antiviral therapies on the thymic output in patients with HIV infection (8, 9), and following stem cell transplantation (10). Furthermore, TREC detection is currently utilized in many countries world-wide for newborn screening of SCID (11, 12).

More recently, we have introduced the use of Ig kappa deleting recombination excision circles (KRECs) of intronRSS-Kde rearrangements to examine B-cell replication (13). Analogous to TRECs, KREC quantification has been incorporated in several newborn screening protocols to detect absence of B cells for identification of X-linked agammaglobulinemia (XLA) and B-cell negative SCID cases (14, 15). The intronRSS-Kde coding joints remain stably present in the genome of mature B cells (16, 17). As a result, the ratio of these genomic coding joints to KREC signal joints is a direct measure for the average number of cell divisions a population of B cells has undergone (13). This accurate quantification has enabled delineation of T-cell dependent and – independent B-cell responses (18), as well as abnormal proliferation of B-cell subsets in common variable immunodeficiency (CVID; a form of PAD) (19) and Down syndrome (20).

In contrast to intronRSS-Kde coding joints, nearly all δREC-ψJα coding joints are removed from the genome in thymocytes during subsequent Vα-Jα gene rearrangements (21, 22). As a result, these cannot be used as a reliable marker for T-cell input, which complicates the use of TRECs to accurately determine T-cell replication history (23). We here present the means to overcome these limitations through the use of a multiplex PCR assay, which detects Vγ-Jγ gene rearrangement coding joints that are stably present in TCRαβ expressing T cells. Together with a newly generated TREC-containing cell line, these can be used to accurately quantify T-cell replication history. We describe accurate replication histories of naive and memory T cell subsets, enhanced T-cell replication with aging and abnormal T-cell replication in PAD patients. Finally, Vγ-Jγ and intronRSS-Kde coding joints can be reliably quantified from Guthrie cards and might form the basis of a second-tier test for absence of TRECs and/or KRECs in neonatal screening for IEI.

MATERIALS AND METHODS

Research Subjects and Ethics

All studies were conducted in accordance with the declaration of Helsinki. Blood samples from adult patients with XLA or genetically-undefined PAD, as well as healthy adults were obtained after written informed consent was provided. Buffy coats were obtained from anonymous donors from the Australian Red Cross. These studies were approved by the human ethics committees of The Alfred Hospital (109/15) and Monash University (MEC# CF15/771 and 2016-0289).

Stored Guthrie cards of 107 Dutch anonymous controls and 84 children with Down syndrome, prepared ∼3–5 days after birth, were collected after obtaining parental consent (24). All Guthrie cards had been stored at room temperature for 3–9 years. This study was approved by the Medical Ethical Committee "METOPP" employed by the Jeroen Bosch Hospital, the Netherlands.

Generation of Control Cell Lines for the TREC-Assay

A human TREC signal joint was PCR amplified in two parts from genomic DNA of thymocytes to introduce a *Bam*HI restriction site 63 bp downstream of the signal joint, and subsequently cloned into the retroviral LZRS-IRES-lyt2 vector (**Figure 1A**). The LZRS-TREC construct was transfected into the Phoenix amphotropic packaging cell line using Fugene-6 (Roche Molecular Biochemicals, Branchbury, NY). Stable high-titer producer clones were selected with puromycin (1 μg/ml). The U698-DB01 pre-B-cell line (13, 25) and the HSB-2 immature T cell line (26) were cultured for several days in RPMI 1640 medium containing 10% FCS and antibiotics before transduction using Retronectin-coated Petri dishes (Takara, Shiga, Japan) and recombinant retrovirus containing supernatant for 2 days, with daily replenishing of retroviral supernatant. Cells expressing mouse CD8 (from the *lyt2* insert) were single-cell sorted using a FACSAriaI cell sorter (BD Biosciences). Individual clones were selected for dim mCD8 expression suggesting a single genomic integration, and subsequently subjected to real-time quantitative PCR to confirm the single-copy integration (see below).

Isolation of T-Cell Subsets From Human Blood

Post-Ficoll mononuclear cells from blood bank donors were stored in 10% DMSO in liquid nitrogen prior to use. Using magnetic bead-based positive selection, CD4+ T cells were separated from thawed samples, followed by positive selection for CD8+ T cells (Dynabeads; Thermo Fisher). Both T-cell fractions were stained with fluorochrome-conjugated antibodies (**Table S1**) prior to sort-purification of four CD4+ and four CD8+ T-cell subsets on a FACSAriaI (BD Biosciences).

DNA Extraction From Full Blood, Cell Lines, T-Cell Subsets, and Guthrie Cards

Genomic DNA was isolated from 200 μl whole blood of adult controls and antibody-deficient patients using a whole blood DNA extraction kit (Sigma-Aldrich) and eluted in 200 μl MilliQ. A genomic DNA Miniprep kit (Sigma-Aldrich) was used to isolate DNA from cultured cell lines and sort-purified T-cell subsets. DNA from 3 millimeter punches of Guthrie cards was isolated using the Sigma Genelute DNA Kit, according to the manufacturer's instructions and eluted in 100 μl MilliQ.

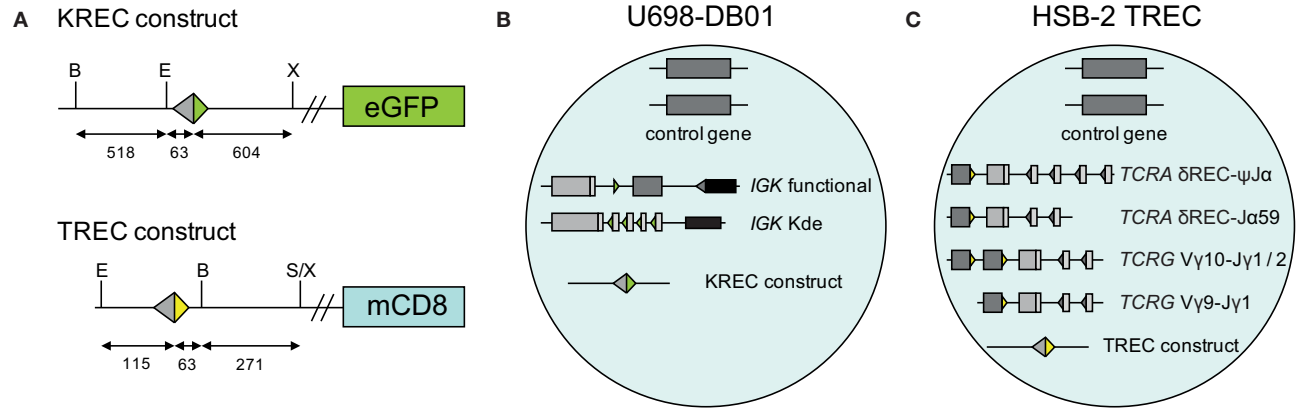

FIGURE 1 | Generation of TREC signal joint containing cell lines. **(A)** Schematic overview of KREC and TREC constructs. Colored triangles depict RSS, fragment sizes (in bp) are depicted below the constructs, restriction sites: B, BamHI; E, EcoRI; S, SalI; X, XhoI. **(B)** Genetic composition of U698-DB01 and **(C)** HSB-2 TREC cell lines.

Real-Time Quantitative PCR (RQ-PCR)

Independent RQ-PCR reactions were performed in duplicate for the albumin, TREC, KREC, intronRSS-Kde, ψJα_germline, and TRG assays. All experiments with whole blood and T-cell subset DNA were performed in a total mixture of 15 μl containing TaqMan GE Mastermix (Thermo Fisher Scientific), 540 nM of each primer (180 nM in case of multiplex mixtures), 60 nM of each 6-FAM/ZEN/Iowa Black labeled probes (Integrated DNA Technologies) and were run on the QuantStudio 6 Flex (Thermo Fisher Scientific). Five microliter of DNA eluate from Guthrie cards were run in RQ-PCR mixtures of 25 μl containing TaqMan Universal MasterMix (Applied Biosystems, Foster City, CA), 900 nM of each primer (300 nM in case of multiplex mixtures), 100 nM of each FAM-TAMRA labeled probe, 0.4 ng BSA, and were run on the StepOnePlus system (Life Technologies). The primers and probes are listed in **Table S2**. Total DNA input per reaction was generally between 30 and 200 ng and only samples with duplicates differing <1 CT were included in the calculations.

Calculations

The difference in CT values between albumin and either the intronRSS-Kde and TRG coding joints or the intronRSS-Kde and δREC-ψJα signal joints were used to calculate the frequencies of cells carrying these rearrangements in unpurified leukocytes. To correct for any technical variation (efficiency) of the independent PCR reactions, the assays were run in parallel on the U698-DB01 and HSB-2 TREC cell lines. As the U698-DB01 cell line contains one intronRSS-Kde coding joint and one signal joint per genome (**Figure 1B**), and the HSB-2 cell line contains one δREC-ψJα signal joint per genome (**Figure 1C**), the frequency of cells in a sample containing these was calculated as follows:

$$2^{\left[\left(CT_{albumin}-CT_{rearrangement}\right)_{sample}-\left(CT_{albumin}-CT_{rearrangement}\right)_{cell\ line}\right]}\cdot 100\%$$

Because the HSB-2 TREC cell line contains two TRG coding joints per genome (**Figure 1C**), the outcome of the equation above was multiplied by 2 to obtain the frequency of T cells rather than the frequency of rearranged TRG alleles per haplotype.

Absolute copy numbers were calculated with the assumption that the DNA content in human cells is 6.6 pg/cell (27, 28). We did not correct for the additional chromosome 21 in patients with Down syndrome (relative weight contribution <2%).

The difference in CT values between the intronRSS-Kde coding and signal joints, and the TRG coding and TREC signal joints are directly related to the replication history of B cells and T cells, respectively. Taking into account the technical variation, the replication histories were calculated as follows:

$$\text{B}-\text{cell replication}: \quad \left(CT_{KREC}-CT_{intronRSS-Kde}\right)_{sample} - \left(CT_{KREC}-CT_{intronRSS-Kde}\right)_{cell\ line}$$

and

$$\text{T}-\text{cell replication}: \quad \left(CT_{TREC}-CT_{TRG}\right)_{sample} - \left(CT_{TREC}-(CT_{TRG}+1)\right)_{cell\ line}$$

As indicated, the formula to calculate T-cell replication corrects for the presence of 2 TRG alleles per genome vs. only 1 TREC allele per genome.

Statistics

Statistical analyses were performed using the Mann-Whitney test for comparing unpaired samples and the Spearman R test for correlation analysis as indicated in the figure legends (GraphPad Prism 8.2.0 for Mac). Correlations were compared using Fisher r to z test. A $p < 0.05$ was considered statistically significant.

RESULTS

Development of a Multiplex TRG Assay to Quantify T Cells in Blood

In contrast to the intronRSS-Kde rearrangement in B cells, the δREC-ψJα rearrangement in T cells is not an end-stage rearrangement, since the coding joint is removed from the genome during subsequent Vα-Jα gene rearrangements

(**Figure 2A**) (23). Consequently, it is not possible to use the δREC-ψJα rearrangement for T-cell quantification or to calculate T-cell proliferation in combination with TRECs. To overcome this limitation, we designed a multiplex RQ-PCR assay to amplify TRG gene rearrangements. This locus was chosen because it is rearranged in nearly all T-cell progenitors, it is both a one-step and an end-stage rearrangement (not deleted from the genome), and carries limited numbers of V and J genes (**Figure 2B**) (29, 30). In the design, detection of the Jγ1.2 gene was omitted as this is specifically used in TCRγδ-expressing T cells, which hardly undergo δREC-ψJα rearrangements (30).

Individual primer combinations were tested for similar efficiencies using genomic DNA from multiple T-cell lines that had rearranged distinct Vγ and Jγ genes (**Table S3**) (31). The final combination was tested on DNA from purified TCRαβ+ and TCRγδ+ T cells (**Figures 2C,D**). In line with previous observations (30), the ψJα gene was deleted in nearly all TCRαβ+ T cells, whereas it was still abundant in TCRγδ+ T cells. The new TRG assay detected 0.98 *TRG* gene rearrangements per TCRαβ+ T cell, whereas only 0.55 in TCRγδ+ T cells. Importantly, the *TRG* counts correlated significantly with absolute T-cell counts, and the intronRSS-Kde coding joints with absolute B-cell counts (Spearman r = 0.3473, $p = 0.0070$, and Spearman r = 0.4172, $p = 0.0010$, respectively; **Figure S1**).

Cell Line Controls for TREC Analysis

In our previous studies, we introduced an intronRSS–Kde signal joint construct into the genome of the U698-M cell line using retroviral transduction (**Figure 1B**) (13). As this cell line already contained one intronRSS–Kde coding joint and two albumin gene copies per genome, it could be used as technical control for the ratio of coding joints and signal joints to study B-cell proliferation, as well as the quantification of B cells in a mixture population. To enable similar technical correction for studies using TRECs, we inserted a ψJα-δREC signal joint construct in the HSB-2 cell line that contains a ψJα-δREC coding joint as well as two Vγ-Jγ coding joints that were amplified by the TRG assay (**Figures 1C**, **2**).

T-Cell Replication in Healthy Controls

Having established the TRG assay and HSB-2 TREC control cell line, these were utilized to examine the replication histories of naive and memory/effector CD4+ and CD8+ T-cell subsets obtained from healthy controls (**Figure 3A**). Within CD4+ T cells, the CD31+ recent thymic emigrants (RTE) had undergone a median of 4.7 cell divisions and naive cells showed 6.5 cell divisions (**Figure 3B**). The antigen-experienced central memory (Tcm) and TemRO T cells, had increased levels, up to 10.8 and 9.5 cell divisions, respectively.

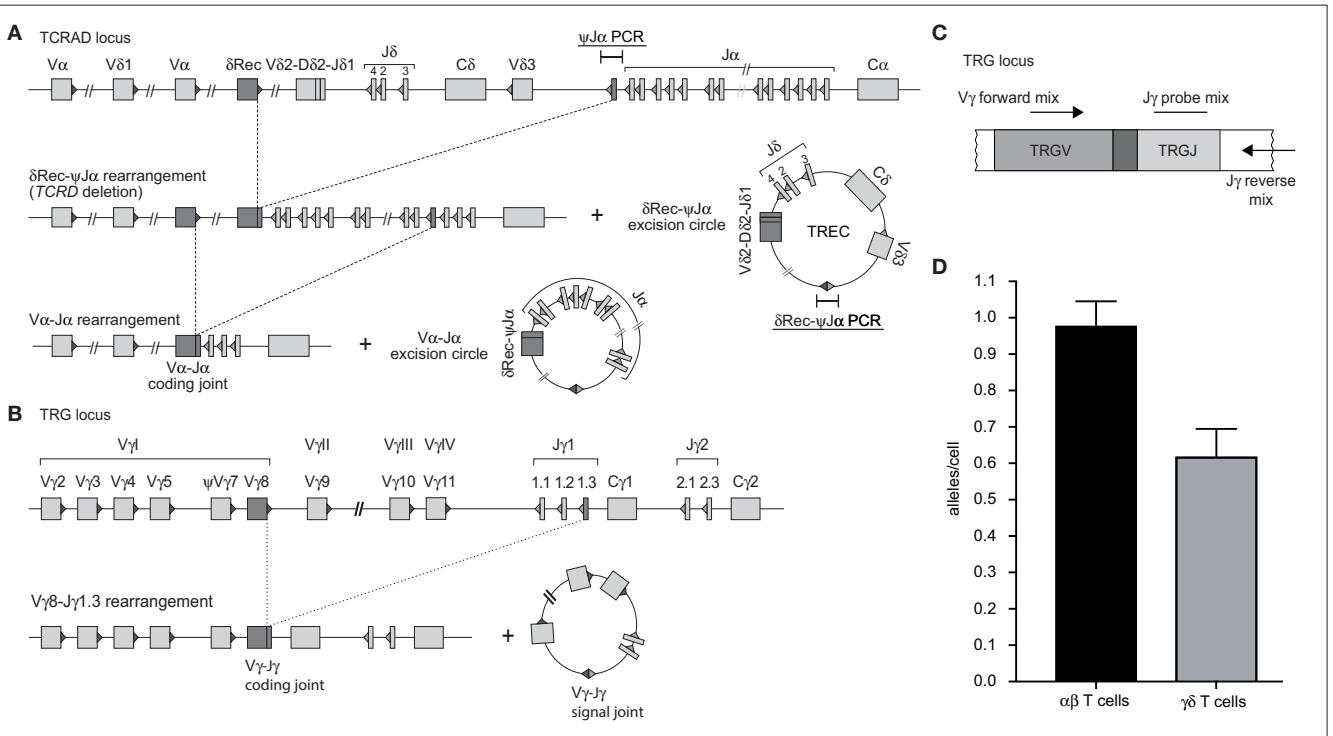

FIGURE 2 | Development of a multiplex TRG PCR to quantify Vγ-Jγ coding joints as a marker for T cells. **(A)** Sequential rearrangements in the *TCRAD* locus. Following V(D)J recombination of *TCRD*, the whole locus is then deleted in the αβ+ T-cell lineage, predominantly by δREC–ψJα rearrangements. The rearrangements give rise to a δREC–ψJα signal joint on an excision circle (TREC) and a δREC–ψJα coding joint in the genome. The coding joint is deleted from the genome by TCRA (Vα-Jα) rearrangements and is then located on an excision circle as well (22, 23). **(B)** Rearrangements of TRG locus resulting in formation of Vγ-Jγ coding joint. **(C)** Schematic overview of the multiplex TGR PCR assay, which contains 4 Vγ forward primers, 2 Jγ reverse primers and 2 Jγ probes (See **Table S2**). **(D)** Mean number of rearranged *TRG* alleles per cell detected by multiplex TRG RQ-PCR assay in purified TCRαβ+ and TCRγδ+ T cells. The reduced detection in TCRγδ+ T cells is the result of our deliberate decision to omit detection of the frequently utilized Jγ1.2 gene in TCRγδ+ T cells.

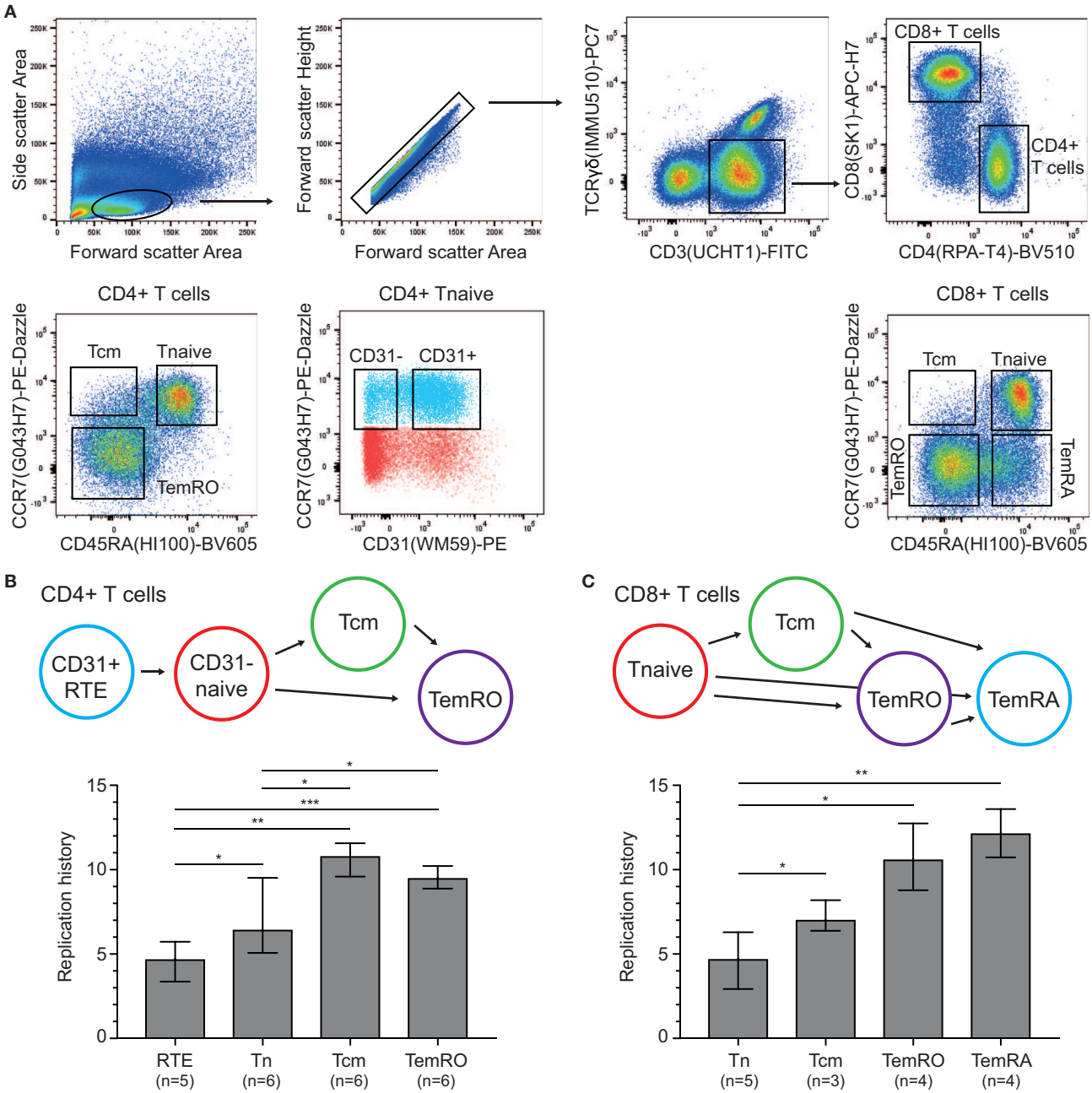

FIGURE 3 | Replication histories of T-cell subsets in healthy controls. **(A)** Flow cytometric representation of T-cell differentiation stages. T-cell subsets were sort-purified based on the indicated gating strategy. **(B,C)** Schematic representation of CD4+ and CD8+ T-cell maturation and replication histories of the purified subsets. RTE, recent thymic emigrants; Tcm, central memory T cell; Tnaive, naive T cell; TemRO and TemRA, terminally differentiated effector memory T cell. The Mann-Whitney U test was used for statistical analysis: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Naive CD8+ T cells showed a similar replication history as CD31+CD4+ naive T cells with 4.7 cell divisions (**Figure 3C**). These levels were significantly higher in Tcm cells with 7.1 cell divisions, and even more in the CCR7- TemRO and TemRA subsets with 10.6 and 12.2 cell divisions, respectively. Thus, in line with B-cell biology (13), the replication history of antigen-experienced T cells is significantly higher than in naive T-cell subsets.

Contributions of Proliferation to Age-Associated Decline in TRECs

We examined TREC, TRG as well as the intronRSS-Kde and KREC assays in a cohort of 59 healthy controls (median age 31 years, range 20–63; **Figure 4**). Similar to Zubakov et al. (32), we found a significant decline in TRECs with age ($r = -0.3552$, $p = 0.0058$). The TRG copy numbers of controls increase with age ($r = 0.2621$, $p = 0.0449$),

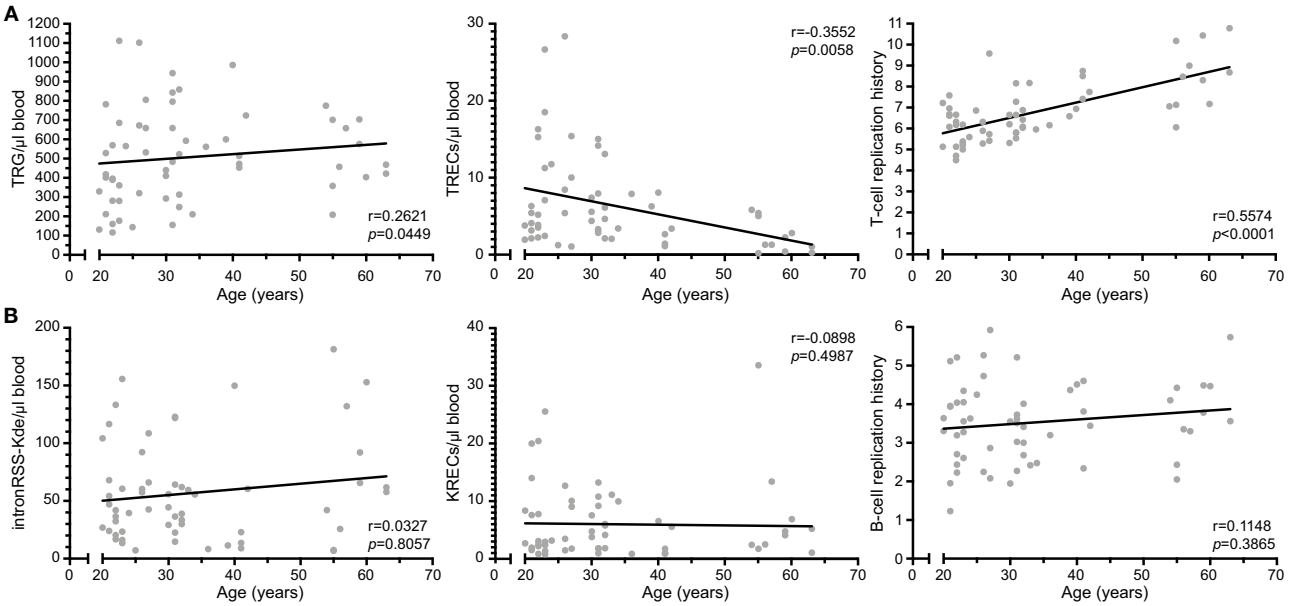

FIGURE 4 | T-cell replication history increases with age. **(A)** Correlation plots of TRG, TRECs and T-cell replication histories as determined from whole blood vs. age of the donor. **(B)** Correlation plots of intronRSS-Kde, KREC and B-cell replication history. Data were obtained from 59 healthy controls. Spearman r was used for statistical analysis.

resulting in a significant increase of cell divisions with age ($r = 0.5574$, $p < 0.0001$).

The copy numbers of intronRSS-Kde, KRECs and B-cell replication history were not significantly correlated with age (**Figure 4B**). Thus, B-cell homeostasis changes minimally with aging, whereas the decline in TRECs with aging for the most part is the result of an increased T-cell replication history.

Assessment of T-Cell Replication in Predominantly Antibody Deficiency

Quantification of TRECs and KRECs has been utilized before to subclassify patients with an antibody deficiency syndrome (33). As no coding joint assays were performed, it remains unclear what the contribution was of proliferation to the findings of abnormally low TRECs and KRECs. Therefore, we here applied our assays to 9 patients with genetically confirmed XLA and 42 patients with PAD (**Table 1**), who were subdivided in two phenotypical categories: infections-only ($n = 14$) and non-infectious complications ($n = 28$) (5, 6).

IntronRSS-Kde and KREC copy numbers in patients with XLA were low or undetectable. The B-cell replication history could therefore only be calculated in 3 patients, which was decreased in one patient (0.3 cell divisions) and normal in the remaining two patients (2.8 and 3.1 cell divisions; **Figure 5A**). Of note, the absolute B-cell counts in these three patients were <1, <1, and 3 per microliter blood. Patients with PAD had normal copy numbers of intronRSS-Kde and lower numbers of KRECs ($p = 0.0085$). Also, their B-cell replication history was higher than in controls (median 4.063 vs. 3.550, $p = 0.0268$). Subsequently, we focused on patients with non-infectious complications and found lower KREC copy numbers as compared to patients with the infections-only phenotype ($p = 0.0453$). Compared to aging in healthy controls, patients with PAD show similar age-related correlations to T- and B-cell replication (**Figure S2**).

The studies of the T-cell compartment in patients with XLA showed normal TRG and TREC copy numbers, as well normal replication history (**Figure 5B**). For patients with PAD, normal TRG copy numbers, but decreased TREC counts and increased T-cell replication history were found as compared to controls (both $p < 0.0001$). There were no differences between patients with and without non-infectious complications.

B- and T-Cell Replication in Dried Blood Spots of Children With Down Syndrome

Newborn screening for T-cell and B-cell lymphopenia is highly sensitive but has a poor specificity (35). Unfortunately, it is currently not possible to determine which patients should be referred urgently and for which patients a repeat screening test should be requested. Quantification of intronRSS-Kde and TRG copy numbers and calculation of the B- and T-cell replication history could potentially be part of second-tier testing in newborns with abnormal screening results to facilitate a risk assessment. In a previous study we showed that newborns with Down syndrome have lower numbers of KRECs and TRECs than healthy controls (24), which we replicated here (**Figures 6A,B**). Since DNA recovery from stored Guthrie cards is less predictable, we corrected our results for DNA input by including a control PCR targeting the albumin gene.

Children with Down syndrome have reduced intronRSS-Kde and TRG copy numbers compared to healthy newborns, which

TABLE 1 | Immunological and clinical characteristics of adults with inborn errors of immunity.

Patient ID	Age at analysis (yr)	Sex	Gene	Mutation	B-cells#/μl blood	T-cells/μl blood	IgG at diagnosis (g/L)	IgA (g/L)	IgM (g/L)	Impaired vaccination response	Infectious complications	Non-infectious complications
X-LINKED AGAMMAGLOBULINEMIA (XLA)												
XLA-01	18	M	BTK	c.1257delG	**<1**	*3,148*	N/A	N/A	N/A	N/A	None	None
XLA-02	21	M	BTK	c.1257delG	**<1**	1,652	N/A	**0.1**	**0.1**	N/A	Pneumonia, bronchiectasis	None
XLA-03	22	M	BTK	c.1257delG	**<1**	1,770	N/A	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia	None
XLA-04	24	M	BTK	c.1257delG	**10**	1,401	N/A	**0.1**	**0.1**	N/A	Sinusitis, bronchiectasis, prostatitis	None
XLA-05 *	24	M	BTK	c.1908+1G>C	**<0.1**	1,497	N/A	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia	Pre-B-ALL
XLA-06	26	M	BTK	c.1257delG	**<0.1**	2,478	N/A	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia, bronchiectasis	None
XLA-07	34	M	BTK	c.862C>T	**1**	**625**	N/A	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia	None
XLA-08	49	M	BTK	c.1559G>A	**3**	**801**	N/A	**0.1**	**0.1**	N/A	Otitis, sinusitis, pneumonia, bronchiectasis	None
XLA-09	59	M	BTK	c.1787+71C>T	**<0.1**	*3,141*	N/A	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia, asthma/COPD, bronchiectasis	None
PAD												
PAD-01	23	F	N/A	N/A	215	1,339	**4.4**	**0.2**	0.4	N/A	Otitis, sinusitis	Enteropathy
PAD-02	23	M	N/A	N/A	446	1,422	**4.9**	**0.3**	**0.3**	N/A	Sinusitis	ITP, AIHA, neutropenia, splenomegaly
PAD-03	24	F	N/A	N/A	**17**	**736**	**<1.4**	**<0.15**	**<0.2**	N/A	Pneumonia, sinusitis,	Arthritis, enteropathy
PAD-04	24	M	N/A	N/A	228	**954**	**1.1**	**<0.1**	**<0.1**	N/A	Pneumonia, otitis, sinusitis, VZV	None
PAD-05	25	M	N/A	N/A	230	1,381	**2.2**	**<0.1**	**0.1**	N/A	Sinusitis, pneumonia	Arthritis
PAD-06	26	M	N/A	N/A	467	*3,355*	N/A	**0.6**	**0.3**	Pneumococcal	Bronchitis, sinusitis, pneumonia	None
PAD-07	27	F	N/A	N/A	**166**	2,008	**3.9**	**0.7**	**0.3**	N/A	Pneumonia, bronchitis	Vitiligo
PAD-08	28	M	N/A	N/A	**37**	**700**	**3.3**	1	0.4	N/A	Bronchitis, pneumonia	Pulmonary nodules, colitis
PAD-09	29	F	N/A	N/A	**144**	1,982	**5.2**	**0.3**	0.5	Normal	Asthma/COPD	None
PAD-10	30	F	N/A	N/A	*1,089*	2,953	**2.2**	**0.4**	0.6	N/A	Sinusitis	Cytopenia
PAD-11	31	F	N/A	N/A	361	**983**	**5.6**	**0.3**	1.3	Normal	Sinusitis, systemic viral infection, giardia	Enteropathy
PAD-12	34	F	N/A	N/A	**46**	**1,083**	**<1**	**<0.1**	**<0.1**	N/A	Otitis, sinusitis, pneumonia, bronchiectasis	GLILD
PAD-13	35	F	N/A	N/A	222	**646**	**2.0**	**0.1**	**0.1**	Pneumococcal	Sinusitis, giardia, asthma/COPD	Enteropathy
PAD-14	37	M	N/A	N/A	**78**	**981**	**4.1**	**0.1**	0.4	Pneumococcal, Hib	Otitis, sinusitis, pneumonia, systemic viral infection, giardia, asthma/COPD, bronchiectasis	Granuloma, enteropathy
PAD-15	37	M	N/A	N/A	**1.3**	**901**	**2.1**	**<0.1**	**<0.1**	Pneumococcal	Pneumonia, asthma, bronchiectasis, chlamydia	ITP, eczema
PAD-16	40	F	N/A	N/A	202	1,038	**5.2**	1.5	0.9	Normal	Sinusitis, pneumonia	None
PAD-17	41	M	N/A	N/A	**35**	1,107	**2.6**	**0.2**	**0.2**	Pneumococcal	Sinusitis, pneumonia, systemic viral infection,	Granuloma, enteropathy
PAD-18	43	F	N/A	N/A	**101**	1,242	**2.0**	**0.3**	**0.2**	Pneumococcal	Sinusitis, pneumonia	Cytopenia
PAD-19	43	F	N/A	N/A	**116**	1,554	**4.2**	3.2	0.6	Pneumococcal	Sinusitis, pneumonia	Inflammatory tracheal stenosis
PAD-20	44	F	N/A	N/A	**177**	**614**	**2.7**	**0.1**	**0.1**	Pneumococcal	Sinusitis, pneumonia, giardia	Splenomegaly, enteropathy
PAD-21	44	M	N/A	N/A	**91**	**834**	**0.6**	**0.0**	**0.2**	N/A	Sinusitis, pneumonia, bronchiectasis	Splenomegaly, lymphadenopathy, granuloma, enteropathy, arthritis
PAD-22	45	M	N/A	N/A	N/A	N/A	**4.7**	2	1.2	N/A	None	HUS

(Continued)

TABLE 1 | Continued

Patient ID	Age at analysis (yr)	Sex	Gene	Mutation	B-cells[#]/μl blood	T-cells/μl blood	IgG at diagnosis (g/L)	IgA (g/L)	IgM (g/L)	Impaired vaccination response	Infectious complications	Non-infectious complications
PAD-23	46	M	N/A	N/A	228	**870**	**0.6**	**0.1**	**0.1**	Pneumococcal	Otitis, sinusitis	None
PAD-24	47	F	N/A	N/A	**84**	1,210	**3.1**	**<0.1**	**<0.1**	N/A	Sinusitis, bronchitis,	Colitis, autoimmunity
PAD-25	50	F	N/A	N/A	N/A	N/A	**5.8**	**0.3**	**0.2**	Pneumococcal	Otitis, sinusitis, pneumonia	None
PAD-26	52	F	N/A	N/A	**96**	**678**	**3.8**	0.9	0.8	N/A	Bronchitis, sinusitis	Pericarditis
PAD-27	52	F	N/A	N/A	193	1,329	**5.2**	**0.8**	1.3	pneumococcal	Sinusitis, pneumonia	None
PAD-28	52	M	N/A	N/A	195	**473**	**3.2**	**0.3**	**0.2**	Hib	Sinusitis, pneumonia	None
PAD-29	54	M	N/A	N/A	245	**775**	**2.9**	**<0.1**	**0.1**	N/A	Sinusitis, pneumonia, asthma/COPD, bronchiectasis	None
PAD-30	54	M	N/A	N/A	**152**	**1,040**	**4.4**	**0.2**	1.0	Pneumococcal, Hib, diphtheria, tetanus	Sinusitis, pneumonia	Solid organ malignancy
PAD-31	54	F	N/A	N/A	373	1,473	**5.4**	1.6	**0.3**	Pneumococcal	Bronchiectasis, sinusitis	Hypothyroidism
PAD-32	54	F	N/A	N/A	**95**	**631**	N/A	**0.2**	**0.4**	N/A	Viral pneumonia, otitis	None
PAD-33	55	F	N/A	N/A	215	1,362	**4.1**	**0.4**	0.8	Normal	Pneumonia	Inflammatory tenosynovitis
PAD-34	55	F	N/A	N/A	**138**	1,214	**4.8**	**0.8**	1.4	Pneumococcal	Otitis, sinusitis, pneumonia	None
PAD-35	56	M	N/A	N/A	**86**	**235**	N/A	**<0.1**	**0.2**	N/A	Pneumonia, hepatitis, colitis	AIHA, ITP, splenomegaly
PAD-36	60	F	N/A	N/A	**119**	1,408	**5.9**	**0.7**	**1.3**	N/A	Sinusitis, otitis, pneumonia	Hashimoto's thyroiditis
PAD-37	62	F	N/A	N/A	204	1,229	**5.6**	1.3	**0.3**	Pneumococcal	Sinusitis	None
PAD-38	66	F	N/A	N/A	**161**	**539**	**3.5**	**0.6**	**0.2**	N/A	Bronchitis, bronchiectasis, asthma	None
PAD-39	67	F	N/A	N/A	191	1,844	**4.6**	**0.6**	*2.9*	Diphtheria	Sinusitis, pneumonia, asthma/COPD	Enteropathy, autoimmunity
PAD-40	73	F	N/A	N/A	**154**	1,345	**3.3**	**0.3**	**0.3**	N/A	Sinusitis, asthma/COPD, bronchiectasis	None
PAD-41	77	F	N/A	N/A	**70**	**553**	**4.2**	**0.8**	1.9	Diphtheria	Systemic viral infection, bronchiectasis, ILD	Solid organ malignancy, auto-immunity
PAD-42	82	M	N/A	N/A	**13**	**727**	N/A	**0.6**	**0.2**	N/A	Otitis, sinusitis, pneumonia, bronchiectasis	Cytopenia
Normal range					190–550	1,090–3,020	6.10–16.2	0.85–4.99	0.35–2.42			

[#]*Values of lymphocyte subsets and immunoglobulin levels below and above normal values are marked in bold and italic font, respectively. *Patient XLA-05 has been described before by van Zelm et al. (34).*

AIHA, auto-immune hemolytic anemia; COPD, chronic obstructive pulmonary disease; GLILD, granulomatous-lymphocytic interstitial lung disease; Hib, Haemophilus influenza B; HUS, hemolytic-uremic syndrome; ILD, interstitial lung disease; ITP, immune-mediated thrombocytopenia; VZV, varicella zoster virus; N/A, not available.

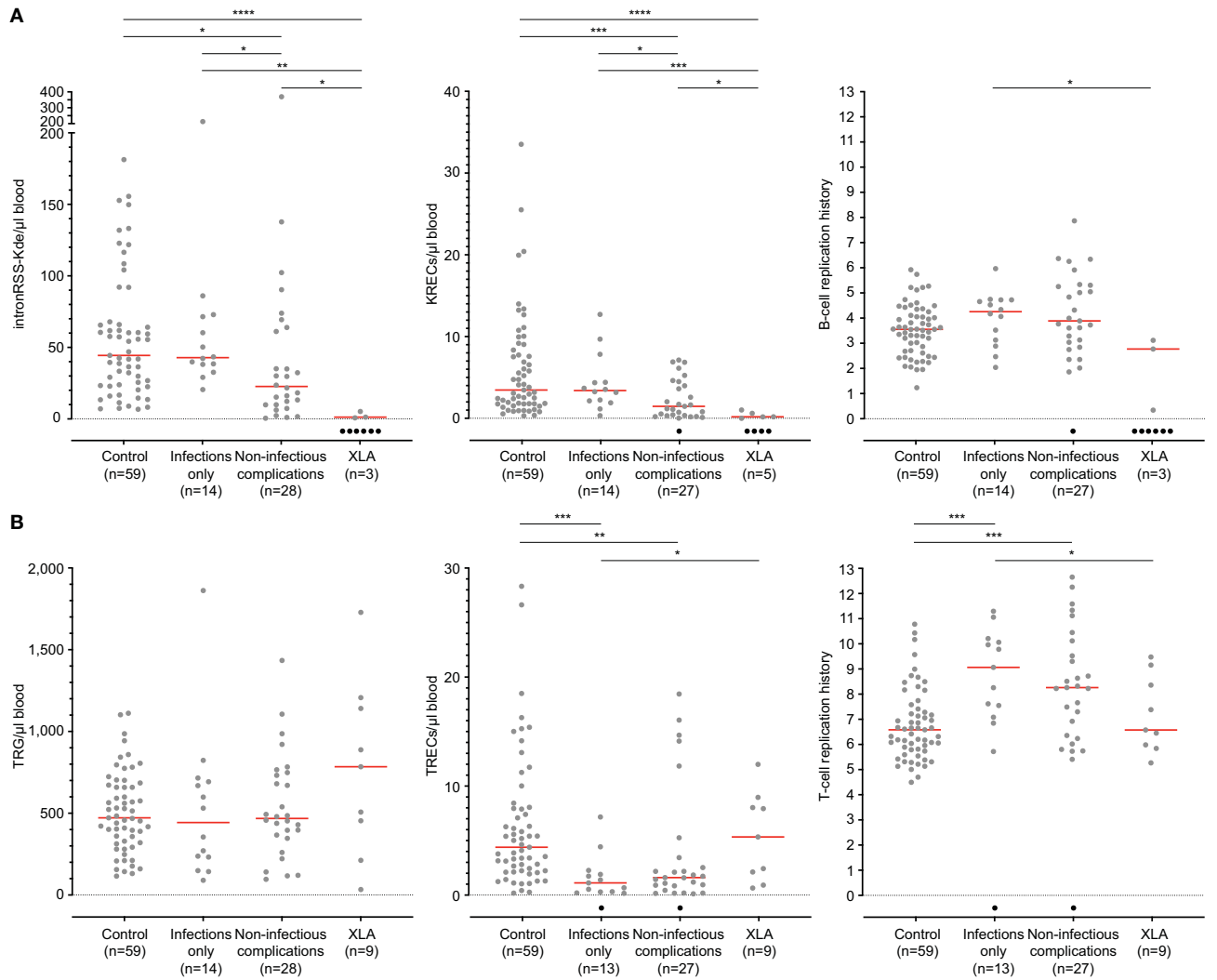

FIGURE 5 | B-cell and T-cell replication histories in PAD patients. **(A)** intronRSS-Kde, KRECs and B-cell replication histories, and **(B)** TRG, TRECs and T-cell replication history for healthy controls and patients with XLA ($n = 9$) and PAD (CVID and hypogammaglobinemia) with infections only ($n = 14$) and non-infectious complications ($n = 28$) as determined from whole blood. Obtained values are shown in gray and undetectable values are shown in black; numbers indicated represent detectable values, and only they were included for statistical analysis with the Mann-Whitney *U*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

is consistent with previous studies showing decreased absolute B- and T-cell counts (**Figures 6A,B**) (36, 37). Subsequently, we calculated the replication histories. In healthy newborns, B cells have undergone a limited number of cell divisions (median 0.57) and T cells show ∼5 cell divisions, which is consistent with results from naive B- and T-cell subsets (**Figure 6C**) (13). In comparison, newborns with Down syndrome showed a slightly increased number of cell divisions in their B-cell compartment than controls (0.885 vs. 0.57, $p = 0.0082$, **Figure 6C**). This could be the result of compensatory proliferation as a result of decreased bone marrow output or increased apoptosis (20, 38). The number of cell divisions for T cells were similar between children with Down syndrome and controls.

In addition to calculation of replication histories, the intron-RSS and TRG assays could be applied as a follow-up test in newborn screening settings to confirm or refute a positive finding with the KREC and TREC assays, respectively. We calculated the sensitivity and specificity of the intronRSS-Kde and TRG assays in our cohort of children with Down syndrome, assuming the KREC and TREC assays as the Gold standard and that values below the 5th percentile of controls were abnormal. Based on abnormal KREC results ("positive test results"), the sensitivity and specificity of the intronRSS-Kde assay were 75 and 80%, and for TRG to confirm an abnormal TREC result these were 45 and 97%. If used as a second tier test, the intron-RSS-Kde assay would confirm the abnormal KREC in 75% of cases, and the TRG assay would do so in 97% of abnormal TREC cases.

DISCUSSION

Here we demonstrate the development and application of a novel multiplex TRG RQ-PCR assay and our TREC containing T-cell

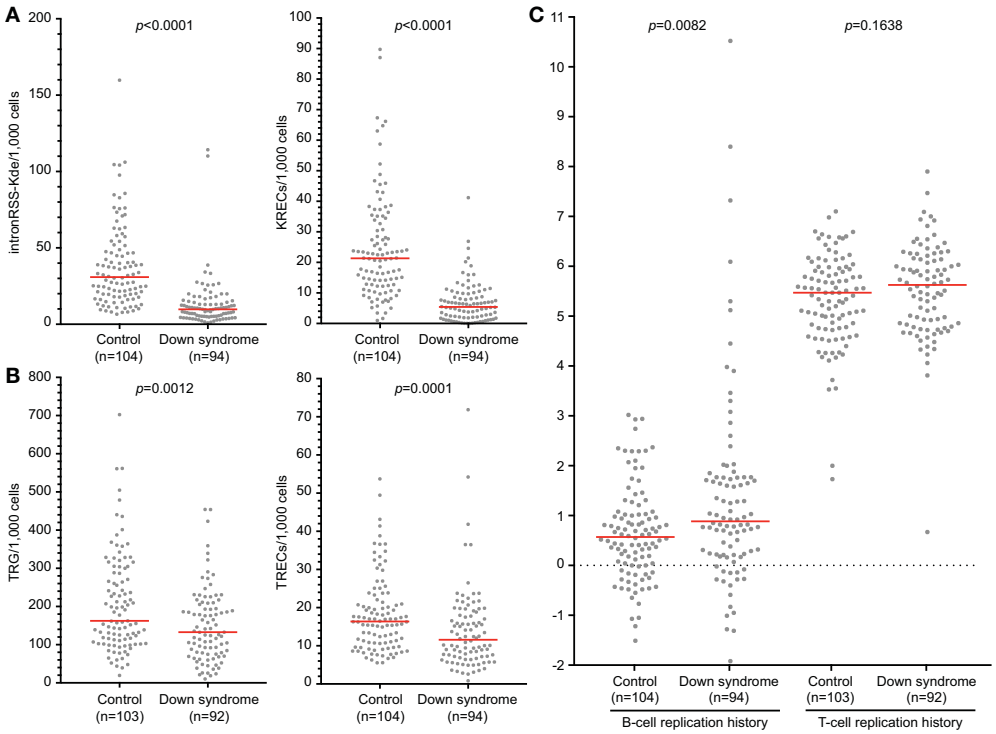

FIGURE 6 | B-cell and T-cell replication histories in neonates with Down syndrome. IntronRSS-Kde and KREC **(A)**, and TRG and TREC **(B)** in healthy neonates and children with Down syndrome, corrected for DNA input as determined from Guthrie cards. **(C)** B- and T-cell replication histories for healthy neonates and children with Down syndrome. The Mann-Whitney *U*-test was used for statistical analysis.

line with biallelic TRG rearrangements enable the quantitative analysis of T-cell replication in normal immunobiology as well as immunodeficiency. By using this approach, we showed that naive T-cell subsets of healthy controls have undergone a limited number of cell divisions (<5) in contrast to antigen experienced effector T cells (>10). Furthermore, we showed that patients with PAD have an abnormal increase in both B- and T-cell replication history. By applying these assays on dried blood spot samples of healthy newborns, we showed that the intronRSS-Kde and TRG coding joint assays showed similar reductions in number as the TREC and KREC signal joint assays. Hence, the coding joint assays could have a role in second-tier testing after screening has identified abnormally decreased TREC and/or KREC levels.

We present a new approach for *ex vivo* quantification of *in vivo* T-cell replication histories. A variety of DNA labeling and intracellular lysine residues techniques have been used to study T-cell replication (39–41). Most previously reported approaches are limited to *in vitro* studies of human T-cell replication, whereas our RQ-PCR assay can be utilized to quantify the *in vivo* replication history. On the other hand, *in vivo* turnover of human T cells can be addressed by deuterium incorporation (42, 43). This has provided new insights into production and longevity of T cells. Ideally, *in vivo* deuterium labeling will be combined with *ex vivo* T-cell replication history analysis to obtain a full picture of T-cell production, longevity and replication in humans.

The TRG assay quantifies coding joints of Vγ-Jγ gene rearrangements and is therefore suitable as a means to quantify T-cell input. As δREC-ψJα coding joints are removed from the genome of developing T cells by Vα-Jα gene rearrangements, these cannot be utilized as a genomic marker (23). Previous studies have used other means to overcome this limitation of the "missing coding joint". The most notable are those in which T cells or subsets have been FACS purified and a general genomic marker was chosen for TREC quantification (e.g., albumin, TRAC or CD3G) (9, 44, 45). Limitations for such an approach are the fact that each cell will contain 2 copies of these genomic markers, whereas δREC-ψJα gene rearrangements are found in less than half of TRA/D loci (30), with the remainder of the TRD loci being deleted by rearrangements involving other genetic elements (22). Moreover, sort-purification of T cells can restrict large-scale analysis of samples as it is labor intensive, costly and requires high cell numbers to yield sufficient material for RQ-PCR. The TRG assay being T-cell specific and detecting just under 1 allele/cell in αβT cells, i.e., very similar to the frequency of δREC-ψJα gene rearrangements. Finally, the combined use with our control cell line enables technical correction for PCR efficiencies, which is needed to accurately determine cell divisions.

As δREC-ψJα gene rearrangements are initiated relatively late during thymocyte development, TREC measurement includes the combined replication history of double positive and single positive CD4 thymocytes, as well as homeostatic proliferation in the periphery, but most likely not that of double negative thymocytes prior to TCRβ selection (30, 44, 45). With the

TRG-TREC approach, we quantified the replication history of naive Th cells to be ∼5 cell divisions for the CD31+ subset and ∼6 for the CD31- subset. This is in line with the ∼6 cell divisions for naive CD4+ T cells from young adults previously reported (44). In addition, it confirms earlier observations that the CD31+ subset is more enriched for TRECs and is likely enriched for RTEs (46, 47).

The replication histories of naive CD8+ and CD4+ T cells were very similar. In contrast, CD8+ Tcm had a much lower average replication history than CD4+ Tcm. Hence, despite their phenotypic similarity, this indicates that different processes underlie their generation, i.e., with distinct levels of proliferation. Alternatively, the CD8+ Tcm subset could be a mixture of memory cells with more naive T cells that have not undergone antigen-induced proliferation.

With normal aging, the total T-cell compartment remains stable in size while naive subsets decrease and effector populations increase (48, 49). This is reflected by stable TRG copy numbers throughout life and decreasing TRECs with higher age (32). As a result, the overall T-cell replication history increases with age.

In our study, we have focused on immunodeficiencies that are characterized by impaired antibody formation. Patients with XLA have strongly decreased or undetectable intronRSS-Kde copy numbers as well as KRECs. We calculated normal B-cell replication histories for 2 out of 3 patients, which is in line with previous findings (50). Normal T-cell replication history was found in patients with XLA. This is consistent with the fact that the BTK protein is normally not expressed in T cells of healthy controls, and T-cell biology does not appear to be affected (51).

Patients with PAD are heterogeneous with regards to their clinical presentations as well as immunologic investigations. These conditions are classically thought to be primarily caused by defective B-cell function. Nonetheless, a significant portion of patients are found to have T-cell abnormalities, especially showing decreased naive T-cell subsets (6, 52, 53). A study from Kamae et al. showed that patients with CVID who have decreased TRECs and KRECs are more likely to develop disease related complications (i.e., infections, autoimmune diseases and malignancies), warranting to consider a diagnosis of combined immunodeficiency (CID) (33). Here, we have shown alterations in the T-cell compartment of patients with PAD that confirm that not only B-cell immunity is affected. Currently, our methods do not allow for classification of patients with PAD in order to identify an underlying (genetic) cause or help to assess the risk for developing non-infectious complications. However, studying the replication history in sorted T-cell subsets might give more insight in the pathways that are affected in patients with PAD and allow for new classification strategies that will assist in clinical management of these patients. As the T-cell abnormalities were not found in XLA patients, it is suggestive that in at least a subset of PAD patients T-cell replication is affected intrinsically, or as a result of additional inflammatory effects not present in XLA patients.

We also demonstrated the potential to quantify the replication history of T-cell subsets. In future studies, this could provide new insights into Th-cell function. For example, Th17 cells which have an important role in the etiology of a variety of inflammatory conditions, including rheumatoid arthritis and inflammatory bowel disease (54, 55). Furthermore, the effects of immunosuppressive treatment could be studied to determine whether normal states can be achieved and serve as a disease monitoring feature. It should be noted that the calculation of T-cell replication will be limited in several disease settings. In cases of severe T-cell lymphopenia, the TREC and/or TRG potentially will not generate a signal, similar to what we observed for the KREC and intronRSS-Kde assays in our patients with XLA. Furthermore, in extreme T-cell lymphoproliferations, TRECs might be extremely diluted and unable to be detected with the TREC assay. This will be especially true in case of monoclonal proliferations, but potentially also in extreme polyclonal proliferations, in parallel to the dilution of KRECs in persistent polyclonal B-cell lymphocytosis (PPBL) (56).

Although newborn screening for T-cell and B-cell lymphopenia is highly effective, at least 80% of newborns with a positive test (reduced TRECs and/or KRECs) turn out to be "false positives" and do not have SCID or XLA. Currently, all abnormal results require drawing of a second blood sample for immunophenotyping. Ideally, a large number of false positives are identified using a second-tier DNA-based test. For example, 22q11 microdeletion syndromes and Down syndrome can be identified in a reliable manner via dried blood spot samples (24, 57). We were able to establish that our intronRSS-Kde and TRG assays generate reliable results on dried blood spot samples. These assays could be applied to samples from children with abnormal TREC and/or KREC screening results, prior to alarming their parents to request a second sample for additional testing.

We here described the development of a novel combined TRG coding joint RQ-PCR and δREC ψJα signal joint RQ-PCR assay with a TREC control cell line, which together allow reliable quantification of *in vivo* T-cell replication history. Our approach has led to new insights in normal T-cell biology, aging and immunodeficiency. Moreover, the high specificity of the TRG and intronRSS-Kde assays to confirm an abnormal TREC and KREC finding in neonates with Down syndrome shows a promise for application of these assays as second tier test in newborn screening. Still, extensive evaluation on large cohorts of neonates with genetically-confirmed SCID or XLA will be required to evaluate if application of these assays could reduce the number of cases in which a second blood sample for verification of abnormal results is required.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MvZ and JvD conceptualized the study and designed experiments. RV, PA, EW, SD, and SB performed experiments. RV and MvZ analyzed and interpreted all data and wrote the manuscript. JB, PC, RS, and EdV established the patient sample collection protocols and included patients into the study. All

authors commented on manuscript drafts and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02084/full#supplementary-material

REFERENCES

1. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, et al. International Union of Immunological Societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *J Clin Immunol.* (2018) 38:96–128. doi: 10.1007/s10875-017-0464-9
2. Cirillo E, Giardino G, Gallo V, D'Assante R, Grasso F, Romano R, et al. Severe combined immunodeficiency–an update. *Ann N Y Acad Sci.* (2015) 1356:90–106. doi: 10.1111/nyas.12849
3. Modell V, Orange JS, Quinn J, Modell F. Global report on primary immunodeficiencies: 2018 update from the Jeffrey Modell Centers Network on disease classification, regional trends, treatment modalities, and physician reported outcomes. *Immunol Res.* (2018) 66:367–80. doi: 10.1007/s12026-018-8996-5
4. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood.* (2008) 112:277–86. doi: 10.1182/blood-2007-11-124545
5. Slade CA, Bosco JJ, Binh Giang T, Kruse E, Stirling RG, Cameron PU, et al. Delayed diagnosis and complications of predominantly antibody deficiencies in a cohort of Australian adults. *Front Immunol.* (2018) 9:694. doi: 10.3389/fimmu.2018.00694
6. Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. *Blood.* (2012) 119:1650–7. doi: 10.1182/blood-2011-09-377945
7. Yamagishi H, Kunisada T, Tsuda T. Small circular DNA complexes in eucaryotic cells. *Plasmid.* (1982) 8:299–306. doi: 10.1016/0147-619X(82)90067-1
8. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature.* (1998) 396:690–5. doi: 10.1038/25374
9. Hazenberg MD, Otto SA, Cohen Stuart JW, Verschuren MC, Borleffs JC, Boucher CA, et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med.* (2000) 6:1036–42. doi: 10.1038/79549
10. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet.* (2000) 355:1875–81. doi: 10.1016/S0140-6736(00)02293-5
11. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol.* (2005) 115:391–8. doi: 10.1016/j.jaci.2004.10.012
12. Routes J, Verbsky J. Newborn screening for severe combined immunodeficiency. *Curr Allergy Asthma Rep.* (2018) 18:34. doi: 10.1007/s11882-018-0783-9
13. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med.* (2007) 204:645–55. doi: 10.1084/jem.20060964
14. Borte S, von Dobeln 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
15. King JR, Hammarstrom L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol.* (2018) 38:56–66. doi: 10.1007/s10875-017-0455-x
16. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of kappa genes in human B cells. *Nature.* (1985) 316:260–2. doi: 10.1038/316260a0
17. Inlay M, Alt FW, Baltimore D, Xu Y. Essential roles of the kappa light chain intronic enhancer and 3' enhancer in kappa rearrangement and demethylation. *Nat Immunol.* (2002) 3:463–8. doi: 10.1038/ni790
18. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood.* (2011) 118:2150–8. doi: 10.1182/blood-2011-04-345579
19. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. *Blood.* (2011) 118:6814–23. doi: 10.1182/blood-2011-06-361881
20. Verstegen RHJ, Driessen GJ, Bartol SJW, van Noesel CJM, Boon L, van der Burg M, et al. Defective B-cell memory in patients with Down syndrome. *J Allergy Clin Immunol.* (2014) 134:1346–53.e9. doi: 10.1016/j.jaci.2014.07.015
21. van Dongen JJ, Wolvers-Tettero IL. Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. *Clin Chim Acta.* (1991) 198:93–174. doi: 10.1016/0009-8981(91)90247-A
22. Verschuren MC, Wolvers-Tettero IL, Breit TM, Noordzij J, van Wering ER, van Dongen JJ. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J Immunol.* (1997) 158:1208–16.
23. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med.* (2001) 79:631–40. doi: 10.1007/s001090100271
24. Verstegen RH, Borte S, Bok LA, van Zwieten PH, von Dobeln U, Hammarstrom L, et al. Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases. *J Allergy Clin Immunol.* (2014) 133:1208–11. doi: 10.1016/j.jaci.2013.10.010
25. Nilsson K, Sundstrom C. Establishment and characteristics of two unique cell lines from patients with lymphosarcoma. *Int J Cancer.* (1974) 13:808–23. doi: 10.1002/ijc.2910130609
26. Adams RA, Flowers A, Davis BJ. Direct implantation and serial transplantation of human acute lymphoblastic leukemia in hamsters, SB-2. *Cancer Res.* (1968) 28:1121–5.
27. Morton NE. Parameters of the human genome. *Proc Natl Acad Sci USA.* (1991) 88:7474–6. doi: 10.1073/pnas.88.17.7474
28. Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R, Jonas U. Quantitation of DNA extracted after micropreparation of cells from frozen and

formalin-fixed tissue sections. *Am J Pathol.* (2000) 156:1189–96. doi: 10.1016/S0002-9440(10)64989-9
29. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia.* (2003) 17:2257–317. doi: 10.1038/sj.leu.2403202
30. Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med.* (2005) 201:1715–23. doi: 10.1084/jem.20042524
31. Sandberg Y, Verhaaf B, van Gastel-Mol EJ, Wolvers-Tettero IL, de Vos J, Macleod RA, et al. Human T-cell lines with well-defined T-cell receptor gene rearrangements as controls for the BIOMED-2 multiplex polymerase chain reaction tubes. *Leukemia.* (2007) 21:230–7. doi: 10.1038/sj.leu.2404486
32. Zubakov D, Liu F, van Zelm MC, Vermeulen J, Oostra BA, van Duijn CM, et al. Estimating human age from T-cell DNA rearrangements. *Curr Biol.* (2010) 20:R970–1. doi: 10.1016/j.cub.2010.10.022
33. Kamae C, Nakagawa N, Sato H, Honma K, Mitsuiki N, Ohara O, et al. Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin kappa-deleting recombination excision circles. *J Allergy Clin Immunol.* (2013) 131:1437–40.e1435. doi: 10.1016/j.jaci.2012.10.059
34. van Zelm MC, Pumar M, Shuttleworth P, Aui PM, Smart JM, Grigg A, et al. Functional antibody responses following allogeneic stem cell transplantation for TP53 mutant pre-B-ALL in a patient with X-linked agammaglobulinemia. *Front Immunol.* (2019) 10:895. doi: 10.3389/fimmu.2019.00895
35. 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) 312:729–38. doi: 10.1001/jama.2014.9132
36. Kusters MA, Gemen EF, Verstegen RH, Wever PC, DE Vries E. Both normal memory counts and decreased naive cells favor intrinsic defect over early senescence of Down syndrome T lymphocytes. *Pediatr Res.* (2010) 67:557–62. doi: 10.1203/PDR.0b013e3181d4eca3
37. Verstegen RH, Kusters MA, Gemen EF, DE Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. *Pediatr Res.* (2010) 67:563–9. doi: 10.1203/PDR.0b013e3181d4ecc1
38. Gemen EF, Verstegen RH, Leuvenink J, de Vries E. Increased circulating apoptotic lymphocytes in children with Down syndrome. *Pediatr Blood Cancer.* (2012) 59:1310–2. doi: 10.1002/pbc.24246
39. Hellerstein MK. Measurement of T-cell kinetics: recent methodologic advances. *Immunol Today.* (1999) 20:438–41. doi: 10.1016/S0167-5699(99)01529-7
40. Sun X, Zhang C, Jin H, Sun G, Tian Y, Shi W, et al. Flow cytometric analysis of T lymphocyte proliferation *in vivo* by EdU incorporation. *Int Immunopharmacol.* (2016) 41:56–65. doi: 10.1016/j.intimp.2016.10.019
41. Quah BJ, Parish CR. The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. *J Visual Exp.* (2010) 44:2259. doi: 10.3791/2259
42. Baliu-Pique M, Verheij MW, Drylewicz J, Ravesloot L, de Boer RJ, Koets A, et al. Short lifespans of memory T-cells in bone marrow, blood, and lymph nodes suggest that T-cell memory is maintained by continuous self-renewal of recirculating cells. *Front Immunol.* (2018) 9:2054. doi: 10.3389/fimmu.2018.02054
43. Borghans JAM, Tesselaar K, de Boer RJ. Current best estimates for the average lifespans of mouse and human leukocytes: reviewing two decades of deuterium-labeling experiments. *Immunol Rev.* (2018) 285:233–48. doi: 10.1111/imr.12693
44. van der Weerd K, Dik WA, Schrijver B, Bogers AJ, Maat AP, van Nederveen FH, et al. Combined TCRG and TCRA TREC analysis reveals increased peripheral T-lymphocyte but constant intra-thymic proliferative history upon ageing. *Mol Immunol.* (2013) 53:302–12. doi: 10.1016/j.molimm.2012.08.019
45. Dion ML, Poulin JF, Bordi R, Sylvestre M, Corsini R, Kettaf N, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity.* (2004) 21:757–68. doi: 10.1016/j.immuni.2004.10.013
46. Kimmig S, Przybylski GK, Schmidt CA, Laurisch K, Mowes B, Radbruch A, et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med.* (2002) 195:789–94. doi: 10.1084/jem.20011756
47. Haines CJ, Giffon TD, Lu LS, Lu X, Tessier-Lavigne M, Ross DT, et al. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. *J Exp Med.* (2009) 206:275–85. doi: 10.1084/jem.20080996
48. Miller RA. The aging immune system: primer and prospectus. *Science.* (1996) 273:70–4. doi: 10.1126/science.273.5271.70
49. Sprent J, Tough DF. Lymphocyte life-span and memory. *Science.* (1994) 265:1395–400. doi: 10.1126/science.8073282
50. Nonoyama S, Tsukada S, Yamadori T, Miyawaki T, Jin YZ, Watanabe C, et al. Functional analysis of peripheral blood B cells in patients with X-linked agammaglobulinemia. *J Immunol.* (1998) 161:3925–9.
51. Smith CI, Baskin B, Humire-Greiff P, Zhou JN, Olsson PG, Maniar HS, et al. Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J Immunol.* (1994) 152:557–65.
52. Moratto D, Gulino AV, Fontana S, Mori L, Pirovano S, Soresina A, et al. Combined decrease of defined B and T cell subsets in a group of common variable immunodeficiency patients. *Clin Immunol.* (2006) 121:203–14. doi: 10.1016/j.clim.2006.07.003
53. Bateman EA, Ayers L, Sadler R, Lucas M, Roberts C, Woods A, et al. T cell phenotypes in patients with common variable immunodeficiency disorders: associations with clinical phenotypes in comparison with other groups with recurrent infections. *Clin Exp Immunol.* (2012) 170:202–11. doi: 10.1111/j.1365-2249.2012.04643.x
54. Abraham C, Dulai PS, Vermeire S, Sandborn WJ. Lessons learned from trials targeting cytokine pathways in patients with inflammatory bowel diseases. *Gastroenterology.* (2017) 152:374–88.e374. doi: 10.1053/j.gastro.2016.10.018
55. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. *Nat Rev Rheumatol.* (2015) 11:415–29. doi: 10.1038/nrrheum.2015.53
56. Berkowska MA, Grosserichter-Wagener C, Adriaansen HJ, de Ridder D, Mirani-Oostdijk KP, Agteresch HJ, et al. Persistent polyclonal B-cell lymphocytosis: extensively proliferated CD27+IgM+IgD+ memory B cells with a distinctive immunophenotype. *Leukemia.* (2014) 28:1560–4. doi: 10.1038/leu.2014.77
57. Lingman Framme J, Borte S, von Dobeln U, Hammarstrom L, Oskarsdottir S. Retrospective analysis of TREC based newborn screening results and clinical phenotypes in infants with the 22q11 deletion syndrome. *J Clin Immunol.* (2014) 34:514–9. doi: 10.1007/s10875-014-0002-y

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ORIGINAL RESEARCH
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First Universal Newborn Screening Program for Severe Combined Immunodeficiency in Europe. Two-Years' Experience in Catalonia (Spain)

Ana Argudo-Ramírez[1†], Andrea Martín-Nalda[2†], Jose L. Marín-Soria[1], Rosa M. López-Galera[1], Sonia Pajares-García[1], Jose M. González de Aledo-Castillo[1], Mónica Martínez-Gallo[3], Marina García-Prat[2], Roger Colobran[3,4], Jacques G. Riviere[2], Yania Quintero[1], Tatiana Collado[1], Judit García-Villoria[1], Antonia Ribes[1] and Pere Soler-Palacín[2]*

[1] Newborn Screening Laboratory, Inborn Errors of Metabolism Division, Biochemistry and Molecular Genetics Department, Hospital Clínic, Barcelona, Spain, [2] Pediatric Infectious Diseases and Immunodeficiencies Unit, Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Immunology Division, Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain, [4] Department of Clinical and Molecular Genetics, Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain

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Mirjam van der Burg, Leiden University Medical Center, Netherlands

***Correspondence:**
Pere Soler-Palacín
psoler@vhebron.net

[†] These authors have contributed equally to this work

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Severe combined immunodeficiency (SCID), the most severe form of T-cell immunodeficiency, can be screened at birth by quantifying T-cell receptor excision circles (TRECs) in dried blood spot (DBS) samples. Early detection of this condition speeds up the establishment of appropriate treatment and increases the patient's life expectancy. Newborn screening for SCID started in January 2017 in Catalonia, the first Spanish and European region to universally include this testing. The results obtained in the first 2 years of experience are evaluated here. All babies born between January 2017 and December 2018 were screened. TREC quantification in DBS (1.5 mm diameter) was performed with the Enlite Neonatal TREC kit from PerkinElmer (Turku, Finland). In 2018, the retest cutoff in the detection algorithm was updated based on the experience gained in the first year, and changed from 34 to 24 copies/μL. This decreased the retest rate from 3.34 to 1.4% (global retest rate, 2.4%), with a requested second sample rate of 0.23% and a positive detection rate of 0.02%. Lymphocyte phenotype (T, B, NK populations), expression of CD45RA/RO isoforms, percentage and intensity of TCR αβ and TCR γδ, presence of HLA-DR+ T lymphocytes, and *in vitro* lymphocyte proliferation were studied in all patients by flow cytometry. Of 130,903 newborns screened, 30 tested positive, 15 of which were male. During the study period, one patient was diagnosed with SCID: incidence, 1 in 130,903 births in Catalonia. Thirteen patients had clinically significant T-cell lymphopenia (non-SCID) with an incidence of 1 in 10,069 newborns (43% of positive detections). Nine patients were considered false-positive cases because of an initially normal lymphocyte count with normalization of TRECs between 3 and 6 months of life, four infants had transient lymphopenia due to an initially

low lymphocyte count with recovery in the following months, and three patients are still under study. The results obtained provide further evidence of the benefits of including this disease in newborn screening programs. Longer follow-up is needed to define the exact incidence of SCID in Catalonia.

Keywords: newborn screening, severe combined immunodeficiency, T-cell receptor excision circles, T-cell receptor, T-lymphocytes, stem cell transplantation

INTRODUCTION

Newborn screening (NBS), initially implemented in the United States in 1961 (1), has been available in most developed countries for decades, originally for the detection and early treatment of phenylketonuria and now for other endocrine and metabolic diseases, mainly using tandem mass spectrometry. DNA-based testing for other diseases has been only recently included in the NBS programs of some countries. Since its initial implementation in Wisconsin in 2008, NBS for severe combined immunodeficiency (SCID) using a T-cell receptor excision circle (TREC) assay has been established worldwide, including in most of the United States (2), Taiwan (3), Israel (4), New Zealand, some Canadian regions (2), Norway (5), and Catalonia (Spain). Previously published results showed an incidence of SCID of around 1 in 56,000 newborns (5, 6) and a high survival rate (92%) after appropriate treatment (2).

SCID, the most severe form of T-cell primary immunodeficiency (PID), includes a group of inherited defects characterized by severe T-cell lymphopenia (TCL). Patients with SCID require prompt clinical intervention to prevent severe life-threatening infections, and several studies have reported significantly improved survival in babies diagnosed at birth (7, 8). Curative treatment is based on hematopoietic stem cell transplantation (HSCT) or gene therapy when available (8). SCID can be screened at birth in a cost-effective way on a large scale through quantification of T-cell receptor excision circles (TRECs) in Guthrie card dried blood spot (DBS) samples (9, 10).

SCID newborn screening can also identify other clinically relevant forms of TCL, which have an overall incidence of 1 in 7,300 newborns (2). These include specific syndromes such as 22q11 deletion (DiGeorge) syndrome, Down syndrome, and CHARGE syndrome among others (11, 12).

TRECs are circular DNA molecules formed by T-cell receptor gene rearrangement during the normal process of T-cell differentiation in the thymus. TRECs do not undergo further replication in dividing cells. Hence, they are stable circular DNA fragments, which are a useful marker of recently formed T-cells, a cell population that is extremely low in newborns with SCID. TREC copy number can be determined using a quantitative PCR-based method with time-resolved fluorescence resonance energy transfer (TR-FRET)-based detection in eluted DNA from routinely collected DBS (9). Kappa-deleting recombination excision circles (KRECs), the circular by-product of B cell immunoglobulin kappa gene rearrangement, have been proposed for a combined TREC-KREC screening approach (13). This could also enable the early detection of patients with severe forms of B cell deficiency, such as X-linked agammaglobulinemia (XLA) (14).

The implementation of screening through TREC assays provides the earliest possible identification and allows for prompt, successful transplantation before infants experience severe infection, organ damage, and, ultimately, death (15). The cost-effectiveness of including SCID in NBS programs has been demonstrated in the United States (16, 17) and Europe (6, 10, 18, 19).

In Catalonia, the NBS program started with phenylketonuria detection in 1969 and congenital hypothyroidism in 1982. Today, around 67,000 newborns per year are screened for 24 diseases (phenylketonuria, congenital hypothyroidism, cystic fibrosis, sickle cell disease, aminoacidopathies, organic acidurias, mitochondrial beta-oxidation disorders, and, lately, SCID). SCID was included in the Catalonian NBS program in January 2017, and data from the first 24 months following its implementation are presented here.

MATERIALS AND METHODS

Population and Data Collection

All consecutive DBS samples received as part of the universal NBS program in Catalonia between January 2017 and December 2018 were analyzed ($n = 130{,}903$). Samples with the following characteristics were excluded: collection time before 44 h or after 7 days of life, transfusions, poor DNA amplification, and poor quality or blood amount. Ultimately, this study was performed in 129,614 newborns.

Demographics (birth date, date of sample collection, parents' origin, newborn sex, gestational age, and birth weight) were electronically collected. Extremely preterm newborns were defined as those with a gestational age <32 weeks, preterm newborns ≥32 and <37 weeks, and term newborns ≥37 weeks. Low birth weight in term babies was defined as <2,500 g and normal birth weight as ≥2,500 g.

From 1 January to 30 June 2017, newborns ($n = 33{,}040$) underwent SCID screening as part of a 6-month prospective implementation pilot study that validated our approach. However, in January 2018, we decided to update the decision algorithm (**Figure 1**), lowering the retest cutoff from 34 to 24 copies/μL. The results from newborns screened in 2018 were then evaluated ($n = 64{,}092$; 63,393 after applying exclusion criteria).

The study was approved by the Government of Catalonia (*Departament de Salut, Generalitat de Catalunya*) for its NBS program. Specific informed consent was obtained from all

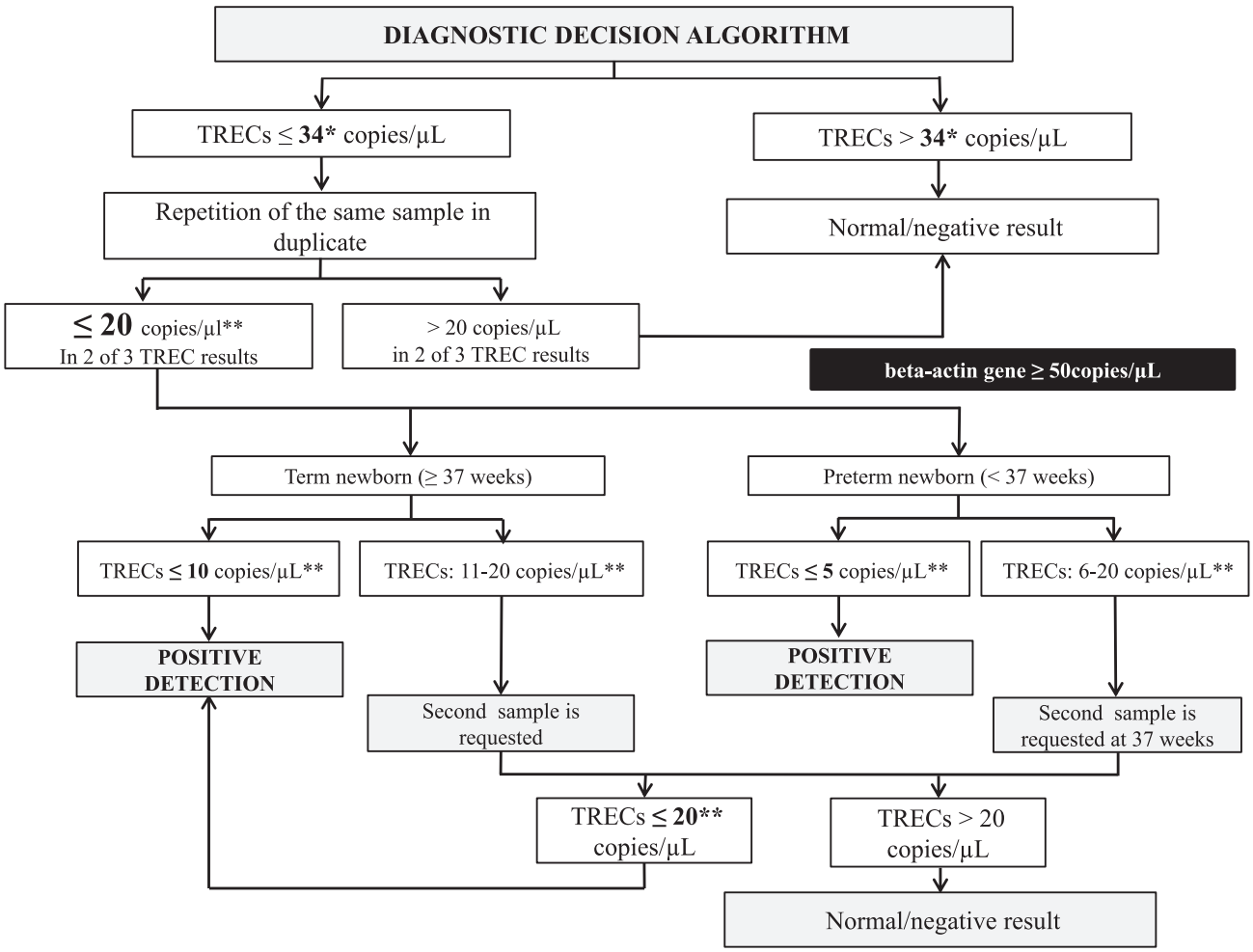

FIGURE 1 | SCID NBS detection decision algorit hm. *The retest cutoff was changed from 34 to 24 copies/μL in 2018. **If beta-actin gene <50 copies/μL a second sample was requested because the sample was considered of unsatisfactory quality. TRECs, T-cell receptor excision circles.

individual participants included in the study whose genetic evaluation was needed.

Sample Testing

Quantification of TRECs in DBS (1.5-mm diameter spot) was performed according to the commercial Enlite Neonatal TREC kit instructions (PerkinElmer, Turku, Finland). The EnLite TREC kit is a combination of PCR-based nucleic acid amplification and time-resolved fluorescence resonance energy transfer (TR-FRET)-based detection. The assay detects two targets simultaneously: TRECs and beta-actin, which is used as the internal control to monitor specimen amplification in each test.

DBS punches were directly inserted in 96-well PCR plates using a Wallac DBS puncher (PerkinElmer). DNA was eluted in the first step, and the second step involved PCR amplification of TRECs and beta-actin as well as hybridization with target sequence-specific probes. The PCR plate was then read in a Victor EnLite fluorometer (PerkinElmer). A full calibration curve with blanks and three DBS calibrators was run in triplicate on each plate. A low-TREC control, a no-TREC control, a high-TREC control, and a blank paper disk (with no blood content) were used as quality controls in each plate. Interpretation of the assay results uses fluorescence counts measured at 615, 665, and 780 nm. Corrected fluorescence counts, TREC responses, and beta-actin responses for all reactions were calculated from the raw fluorescence counts. Calibrator responses were fitted against the ArcSinh transformed concentrations (copies/μL) using unweighted linear regression. The Enlite software generated a calibration curve and the sample and DBS control concentrations (copies/μL) in each run.

Definition and Interpretation of Results

Before implementing the protocol, the method was verified (repeatability, reproducibility, limit of detection, limit of quantification, sensitivity, and specificity) and these parameters were successfully compared with those stated in the kit insert (**Supplementary Material S1**). In addition, available NBS

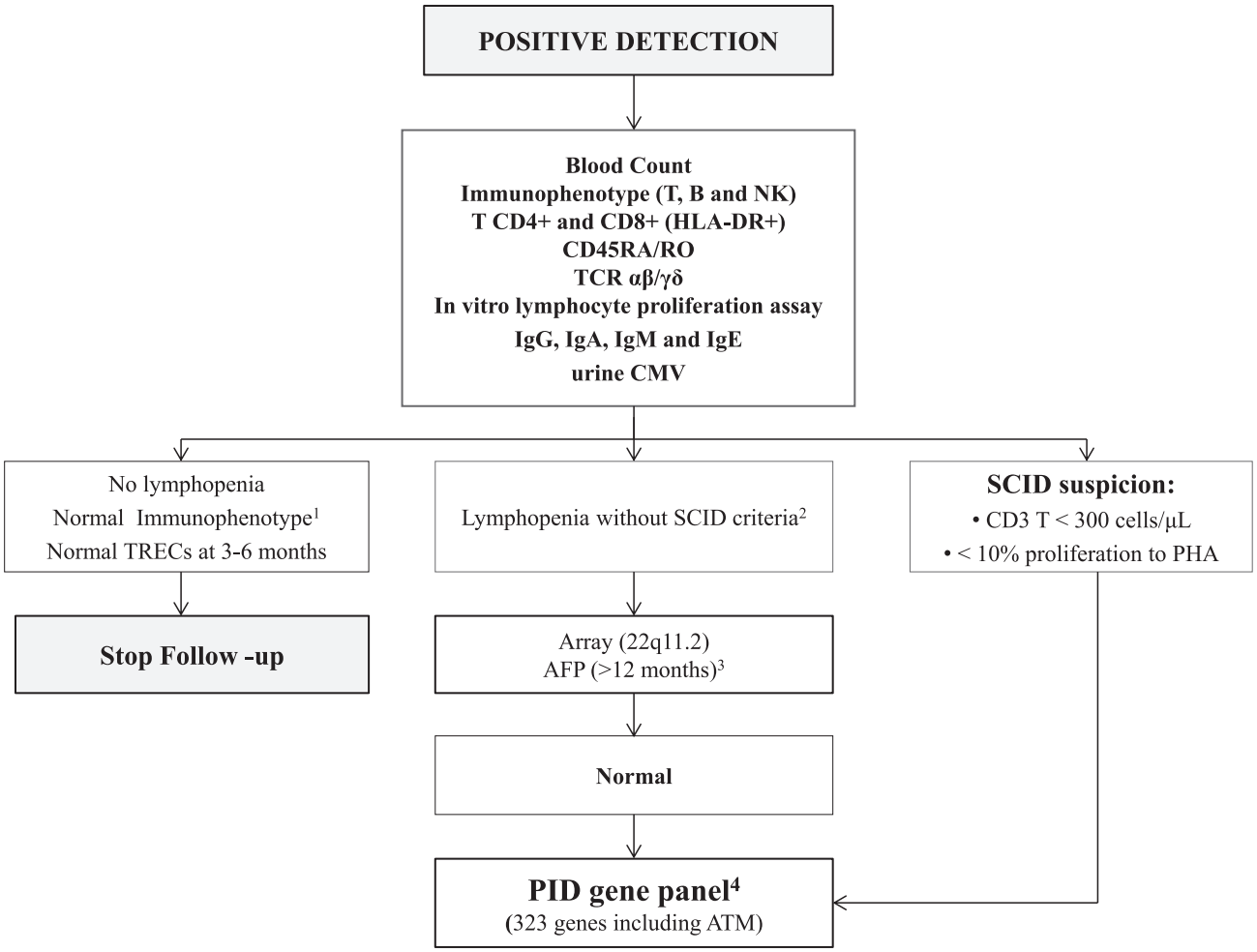

FIGURE 2 | Immunological and genetic protocol in positive cases. [1]Flow cytometry protocols are shown in **Supplementary Material S5**; [2]Lymphopenia and SCID criteria are shown in **Supplementary Material S2**; [3]AFP values are not reliable before 12 months of age; therefore, AFP was only studied in patients older than one year lacking an alternative diagnosis; [4]The list of 323 PID genes is shown in **Supplementary Material S3**. AFP, alpha-fetoprotein; ATM, ataxia telangiectasia; B, B-cells; CMV, cytomegalovirus; Ig, immunoglobulin; NK, natural killer cells; PID, Primary immunodeficiency; SCID, severe combined immunodeficiency; T, T-cells; TCR, T-cell receptor; TRECs, T-cell receptor excision circles.

samples from children with a known SCID diagnosis in Catalonia in the last 5 years were analyzed as positive controls ($n = 6$; median, range TREC copies/μL: 2, 2–4), as well as five other positive samples from the SCID Newborn Screening Quality Assurance Program-Proficiency Testing Program provided by the CDC (Centers for Disease Control and Prevention, Atlanta, USA).

After reviewing the decision algorithms from other NBS programs with previous experience of this disease, we decided to start the pilot study with the algorithm used by Audrain et al. (6), which had a threshold of >34 copies/μL (**Figure 1**).

Samples whose TREC value was ≤34 copies/μL were retested in duplicate. If two of the three values were ≤20 copies/μL, a second sample was requested. Samples with TRECs ≤5 copies/μL (preterm infants) or ≤10 copies/μL (term newborns) in the first sample (both with beta-actin gene ≥50 copies/μL), as well as analyses with TRECs ≤20 copies/μL in the second sample, were considered as positive detection (retest cutoff changed from 34 to 24 copies/μL in 2018). These positive detections were notified to the SCID Clinical Reference Unit (SCID-CRU) to initiate clinical and immunological evaluation.

The retest after the first sample rate (retest rate), requested second sample rate, and SCID-positive detection rate (positive detections) were calculated. Based on these results, the algorithm was reevaluated. Validation of the results was carried out using Specimen Gate (Perkin Elmer) and Nadons (Limit4, Barcelona, Spain) software.

Initial Clinical and Immunological Assessment at SCID-CRU

Within the first 7 days after detection, all positive cases were referred to SCID-CRU, where clinical and immunological assessment was performed per protocol (**Figure 2**). Complete family and medical histories were recorded, and

a meticulous physical examination was carried out. In addition, psychological support was offered to parents, starting with this first visit.

Several tests were performed: T, B, and NK cell immunophenotyping, expression of CD45RA/RO, TCR αβ, and γδ, HLA-DR+ expression on T lymphocytes, *in vitro* lymphocyte proliferation, and immunoglobulins including IgE. SCID criteria (**Supplementary Material S2**) were defined as reported by Kwan et al. (2). In cases of non-SCID lymphopenia, a consultation was scheduled with the geneticist for clinical evaluation and array-based comparative genomic hybridization (CGH-array) studies. If these results were normal, a genetic panel covering most PID was performed. We used a custom-designed next-generation sequencing (NGS)-based panel that targets 323 genes (**Supplementary Material S3**), including most of the known PID-causing genes according to the 2017 IUIS (International Union of Immunological Societies) classification, and other genes recently described as causing PID (20). This panel has been successfully used in our laboratory over the last few years for genetic diagnosis of PID (21).

Statistical Analysis

Statistical analyses were performed using SPSS software version 23.0 (SPSS Inc., Chicago, Illinois). Given the non-parametric distribution of the data, the Mann–Whitney *U* or Kruskal–Wallis test were used for group comparison analyses (25 and 75th percentiles in brackets). Two-tailed statistical analysis was performed, and differences were considered statistically significant at $p < 0.05$.

RESULTS

Demographics

Of the 129,614 newborns screened after applying the exclusion criteria (initial $n = 130,903$), 51.5% were male and 48.5% were female. The median age at sample collection was 50 h of life (interquartile range [IQR], 49–60). Median gestational age was 39 weeks (38–40): 0.6% were extremely preterm newborns ($n = 830$), 5.9% preterm ($n = 7,561$), and 93.5% term newborns ($n = 121,223$). Median birth weight in term newborns was 3,300 g (3,010–3,590) (**Table 1**).

Median TREC value in the study population was 104 copies/μL (IQR: 68–162), with 20 copies/μL being the 0.7th percentile. Median TREC values showed statistically significant differences in relation to the patients' sex, gestational age, and birth weight (only assessed in term babies). Median TREC values were 98 (64–152) and 111 (73–173) in males and females, respectively ($p < 0.05$). In relation to gestational age, the median TREC value demonstrated a progressive increase: 70 (43–127), 96 (62–155), and 105 (68–162) copies/μL in extremely preterm, preterm, and term newborns, respectively ($p < 0.05$) (**Supplementary Material S4**). Regarding birth weight in term newborns, median TREC was 94 (61–145) and 105 (69–163) copies/μL in low and normal birth weight babies, respectively ($p < .05$) (**Table 1**).

TABLE 1 | Demographic data and TREC values in the study population.

Sample size	$n = 129,614$[a]	TRECs, copies/μL, median (IQR)	p-value[c]
All newborns		104 (68-162)	–
Sex			
Male	51.5% (66, 751)	98 (64–152)	<0.05
Female	48.5% (62, 863)	111 (73–173)	
Sample collection, hours, median (IQR)	50 (49–60)	–	–
Gestational age, weeks, median (IQR)	39 (38–40)		
Extremely preterm, <32 weeks, % (*n*)	0.6% (830)	70 (43–127)	<0.05
Preterm, 32-36 weeks, % (*n*)	5.9% (7,561)	96 (62–155)	
Term, ≥37 weeks, % (n)	93.5% (121, 223)	105 (68–162)	
Birth weight in term newborns[b], grams, median (IQR):	3300 (3,010–3,590)		
Low birth weight, % (*n*)	3.4% (4,385)	94 (61–145)	<0.05
Normal weight,% (*n*)	96.6% (114, 777)	105 (69–163)	

[a]*Sample size after applying exclusion criteria;* [b]*Term newborns (n = 121, 223).* [c]*Mann-Whitney U or Kruskal-Wallis test were used for group comparisons (p-value calculated at a 5% significance level). IQR, interquartile range.*

Cutoff Values and Rates

The overall results from 2017 to 2018 were retest rate 2.4%, requested second sample rate 0.23%, and positive detection rate 0.02% (**Figure 3**).

With the application of the initial TREC retest cutoff of 34 copies/μL (3rd percentile in the 2017 population), results from the 66,214 samples analyzed during 2017 were as follows: retest rate 3.34% ($n = 2212$), requested second sample rate 0.21% ($n = 138$), and positive detection rate 0.02% ($n = 16$).

After reducing the retest cutoff to 24 copies/μL (1st percentile), the retest rate was 1.4% ($n = 898$). The detection cutoff of 20 copies/μL remained the same (0.7th percentile), and the requested second sample and positive detection rates were similar to those of 2017.

Positive Detections

There were 30 positive detections in the 129,614 screened newborns: 40% ($n = 12$) were detected in the first sample and 60% ($n = 18$) in the second, with 50% ($n = 15$) of detections occurring in males. There was no history of maternal immunosuppression in any of the positive cases.

Newborns with positive detection were referred to the SCID-CRU per protocol. The various diagnoses in the 30 patients were as follows (**Figure 4**): SCID (1/30), partial 22q11 DiGeorge syndrome (5/30), idiopathic lymphopenia (3/30), chylothorax (2/30), prematurity (2/30), and Down syndrome (1/30). Laboratory and clinical data of non-SCID TCL patients are described in **Table 2**. Nine patients were considered to have false-positive results (i.e., initially low TRECs and normal

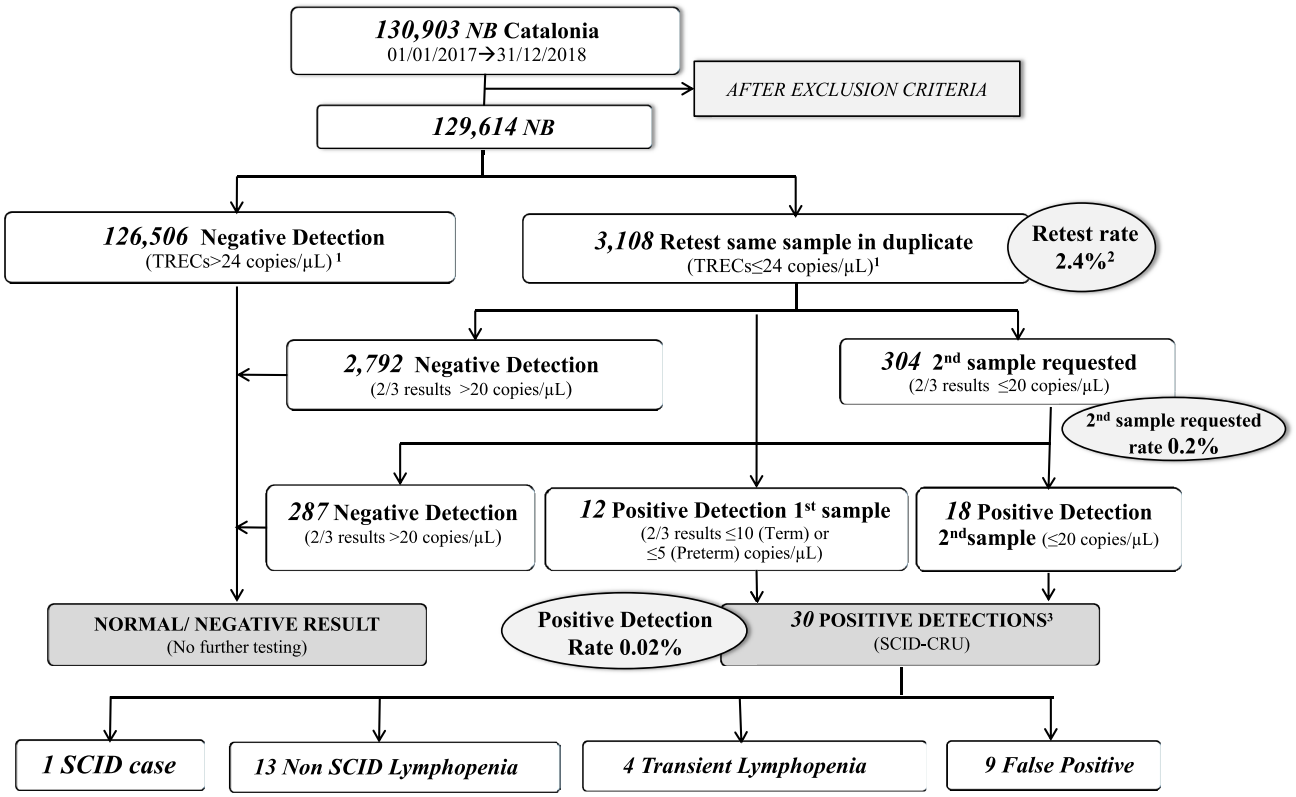

FIGURE 3 | Results of SCID NBS in Catalonia over the study period (2017–2018). [1]2018 cutoff (2017 cutoff = 34 copies/μL); [2]2017+2018-Retest rate 2017-Retest rate = 3.34% (cutoff = 34 copies/μL); 2018-Retest rate = 1.4% (cutoff = 24 copies/μL); [3]Three patients are currently under study. NB, newborns; SCID, severe combined immunodeficiency; SCID-CRU, SCID Clinical Reference Unit.

lymphocyte count, with normalization of TRECs between 3 and 6 months of life), four patients had transient lymphopenia (i.e., initially low TRECs and low lymphocyte count, with recovery in the following months), and three patients are currently under study (SCID excluded, but requiring additional TREC testing at 3 and 6 months of life). The incidence of clinically significant non-SCID TCL was 1 case in 10,069 newborns (43% of positive detections).

Thus, the incidence of SCID was 1 in 130,903 newborns. The SCID patient was a Caucasian male of 35 weeks' gestation with 0/0 TREC copies/μL in DBS. Lymphocyte count was 0.4 $\times$ 10^9/L with a T-B- NK+ phenotype and an absent *in vitro* proliferative response to mitogens. Whole exome sequencing was performed, but no causative mutations were found. However, a variant of uncertain significance was identified in the *LIG1*gene. The patient underwent HSCT at the age of 2 months of life using reduced intensity conditioning, with a good clinical outcome and immunological reconstitution.

DISCUSSION

This study reports the Catalonian NBS experience for SCID detection based on TREC quantification, and enables detection of non-SCID lymphopenic conditions in addition to SCID. After a 2-year experience and more than 130,000 newborns screened, one SCID patient and 13 other conditions were identified. In addition, we were able to consolidate our detection strategy and the characterization, diagnosis, and follow up protocol for positive cases.

As a pioneer initiative in Europe, we encourage the definition of a single technical approach for SCID newborn screening to facilitate future comparative studies between different countries. With this in mind, we decided to implement the commercially available EnLite Neonatal TREC kit (Perkin Elmer, Turku, Finland), which has been validated elsewhere and has shown comparable median TREC values between different studies (4, 6, 22, 23). This kit allows for DNA elution and gene amplification in a single process, as well as standardization and reproducibility according to ISO 15189 standards. With the aim of minimizing the previously described inter-lot variability (6), we established a retest cutoff higher than the request second sample cutoff, which provided a range between the retest and detection cutoffs. The initial retest cutoff was 34 copies/μL (3rd percentile), which was changed to 24 copies/μL (1st percentile) in 2018, whereas the request second sample cutoff was maintained at 20 copies/μL.

The initial retest cutoff of 34 copies/μL (3rd percentile) led to a retest rate of 3.34%, which was considered too high in comparison with data from other authors (24). Hence, we

then evaluated the first percentile from our population (24 copies/μL) as a new retest cutoff. We found that 1,477 of 1,478 babies with initial TREC values between 34 and 25 copies/μL were normal: 1,455 (98.4%) after duplicate repetition and 22 (1.6%) after a second DBS analysis. Only one baby within this range was referred for study in the SCID-CRU and the diagnosis was not a case of SCID, the objective of this screening. Following this analysis, we decided that it was reasonable to switch to 24 copies/μL as the TREC repeat cutoff, implemented since 2018. That change reduced the repetition rate to 1.4%, now comparable to the rate described by others (24) and led to a reduction in the screening cost per patient.

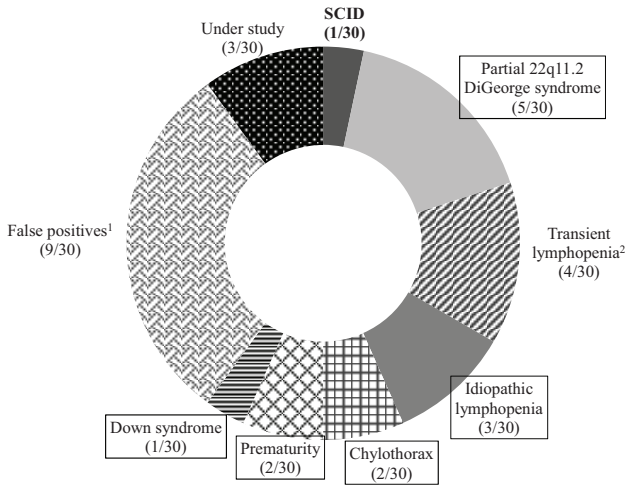

FIGURE 4 | Final diagnoses in patients testing positive in Catalonia (2017–2018). [1]False-positives due to initially normal lymphocyte count with normalization of TRECs between 3 and 6 months of life; [2]Transient lymphopenia due to initially low lymphocyte count with recovery in the following months. Final diagnosis of the 13 non-SCID lymphopenic patients are now boxed. SCID, severe combined immunodeficiency.

Our internal algorithm showed a second sample request rate and positive detection rate of 0.2 and 0.02%, respectively, in keeping with the rates in previous studies, as summarized in the review by van der Spek et al. (24). In addition, the percentiles of our population corresponding to each cutoff are comparable to those of other diseases included in our program, such as congenital hypothyroidism, phenylketonuria, and other metabolic diseases. Of note, the method was unable to adequately quantify beta-actin copies in some of the external quality control samples from the CDC program, a shortcoming that may be related to PCR inhibition, as was suggested by the CDC itself. Nonetheless, this limitation did not affect the results of SCID screening.

Several studies have used combined TREC and KREC screening assays to simultaneously detect T- and B-cell defects (18, 25), including a pioneer study performed in Spain by de Felipe et al. (26), but we decided to focus on classical SCID patients who completely fulfill the NBS criteria, in accordance with the experience in the United States (2, 5). Although

TABLE 2 | Clinical and laboratory data in non-SCID TCL patients.

Patient	Gestational age	Sex	Prenatal diagnosis	TREC levels (copies/μL)	Lymphocyte count, (x10^9/L)*	Lymphocyte subsets:T cells/B cells/NK cells (x10^9/L)**	Lympho-proliferationassay	Clinical features at birth	Diagnosis
1	40	Female	No	17	4.2	2.1/19.9/1.1	Normal	None	22q11.2 DS
2	40	Female	No	5	5.4	1.6/1.1/2.3	Normal	None	22q11.2 DS
3	34	Female	Yes	5	2.6	1.1/0.6/0.7	Normal	Congenital heart disease	22q11.2 DS
4	39	Female	Yes	17	2	1.9/1.4/2	Normal	Congenital heart disease and hypocalcemia due to hypoparathyroidism	22q11.2 DS
5	41	Female	No	17	1.7	1.2/0.04/0.3	Normal	None	22q11.2 duplication
6	40	Male	No	14	3.7	2.2/0.6/0.6	Normal	None	Idiopathic lymphopenia
7	40	Male	No	7	3.3	1.7/0.6/0.7	Normal	None	Idiopathic lymphopenia
8	35.2	Male	No	6	1.7	2/0.2/0.2	Normal	None	Idiopathic lymphopenia
9	34	Female	Yes	6	7.8	NA	Normal	Congenital Chylothorax	Chylothorax
10	34	Female	Yes	4	7.4	NA	Normal	Hydrops fetalis	Chylothorax
11	32	Male	No	7	2.7	2.9/0.8/0.2	Normal	None	Prematurity
12	29	Male	No	18	4.4	2.9/1/0.2	Normal	None	Prematurity
13	31	Female	No	15	3	NA	Normal	Esophageal atresia	Down syndrome

*Reference value: 3.4–7.6; **Reference values: T-cells: 1.8–5.9; B-cells: 0.6–1.9; NK-cells: 0.1–1.3. CGH, comparative genomic hybridization; DS, deletion syndrome; NA, not available; NBS, newborn screening; SCID, severe combined immunodeficiency; TREC, T-cell receptor excision circle.*

inclusion of KREC values to the TREC assay would enable the detection of patients with hypomorphic mutations leading to a leaky SCID phenotype and delayed-onset adenosine deaminase (ADA) deficiency (14, 25, 27), it might also result in an increase in false-positive testing and higher recall and retest rates.

Our data support the previously reported influence of gestational age and sex on TREC values (27–30). Thus, in the case of low values in preterm babies or low-birth-weight term newborns (i.e., TREC values above 5 copies/μL in preterm babies and 10 copies/μL in term newborns), cautious evaluation and a request for a second sample are needed at term and when birth weight reaches >2,500 g, respectively. To our knowledge, there is no explanation for the significantly higher TREC values seen in female newborns; however, these differences have been described previously in a cohort study reported by Rechavi et al. (4) Further studies may provide data to define the reason for these differences and their clinical implications, if any.

With regard to gestational age, median TREC levels in our cohort rose significantly from 28 to 32 weeks gestation in accordance with T-cell maturation in this period, a wider period of time than those reported by other authors (4, 18, 26) (**Supplementary Material S4**).

Overall, there were 30 positive detections in the 129,614 newborns screened (130,903 before applying the exclusion criteria). All were referred to the SCID-CRU per protocol. Nine patients were considered to have false-positive results due to initially normal total lymphocyte and CD3+ counts with normalization of TRECs between 3 and 6 months of life, whereas four others had transient lymphopenia at the beginning with recovery in the following months, and three are currently under study [none of the three patients under study met the SCID criteria, as defined by Kwan et al. (2)].

The diagnosis of the T-B-NK+ SCID patient permitted timely referral to a specialized treatment center for HSCT, after which T and B lymphocyte counts normalized with fully reconstituted function, eliminating the need for IgG supplementation at 1 year of age.

The incidence of SCID found in this study (1 in 130,903) is lower than the reported rates from other areas (2, 4, 24). However, we anticipate that a more robust incidence will be established when the population analyzed reaches 200,000 newborns. In this line, a retrospective study in our region demonstrated an incidence of classically diagnosed SCID of 1 in 57,000 newborns (unpublished data). In addition to the SCID patient, 13 infants with non-SCID TCL were identified, yielding an incidence of 1 in 10,069 newborns (43% positive detections). This is similar to the rate reported by others (31). The diagnostic distribution was also comparable. It is worth mentioning that early detection of patients with 22q11.2 deletion syndrome allowed a prompt intervention to anticipate complications, as reported by our group (Mol Genet Genomic Med, accepted).

Of note, one of the strengths of our SCID NBS approach is the psychological support offered to the families. A positive result in any NBS program is a cause of concern for parents at this vulnerable time because of an unfamiliarity with these illnesses, the young age of the patients, and the stress on families after a birth. Therefore, in neonates initially testing positive, a psychologist provided support starting from the first contact with the family in a joint visit with the pediatric immunologist. The psychologist's task was to guarantee that parents were able to understand and assimilate the information about the possible diagnosis and the process to follow in the event that it was confirmed. The family was offered a space for containment and support for the impact of the diagnosis, and strategies were activated to control anxiety. The psychologist maintained contact to ensure adherence to the treatments prescribed and compliance with the control visits and tests. Support was intensified in the most complicated moments, such as hospital admission and isolation, and parents were accompanied in all phases related to transplantation. Several cases of parental anxiety were detected and appropriately managed. In the yearly evaluation, families rated the psychological support in our NBS program very favorably.

The main limitation of this study is the relatively small number of newborns screened, although the similarity between our results and those reported in larger cohorts supports their validity. In addition, retrospective data show an incidence of SCID in our region similar to that of other Western European countries (6, 18) and the United States (5).

To conclude, TREC quantification in DBS for SCID detection has been satisfactorily implemented in the Catalonian NBS program. Retesting, requested second samples, and positive detection rates were optimal with the current algorithm and similar to published data. Our results provide further evidence to support the inclusion of SCID in NBS programs in other regions and countries. Longer follow-up is needed to define the exact incidence of SCID in Catalonia.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Ético from Agència de Salut Pública (Catalonia). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AA-R, AM-N, and PS-P designed the study. YQ and TC collected the data and participated in the analytical tools. AA-R and JM-S organized the database. AA-R and RL-G performed the statistical analysis. Results were analyzed and interpreted by AA-R, AR, AM-N, and PS-P. AA-R, AM-N, and PS-P wrote the first draft of the manuscript. MG-P, MM-G, JR, RC, SP-G, JG, JG-V, and JM-S wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02406/full#supplementary-material

REFERENCES

1. Guthrie, R. Blood screening for phenylketonuria. *JAMA*. (1961) 178:863. doi: 10.1001/jama.1961.03040470079019
2. 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) 312:729–38. doi: 10.1001/jama.2014.9132
3. 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) 114:12–6. doi: 10.1016/j.jfma.2012.10.020
4. 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. doi: 10.3389/fimmu.2017.01448
5. Puck JM. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. *Immunol Rev*. (2019) 287:241–52. doi: 10.1111/imr.12729
6. 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). *J Clin Immunol.* (2018) 38:778–86. doi: 10.1007/s10875-018-0550-7
7. Brown L, Xu-Bayford J, Allwood Z, Slatter M, Cant A, Davies EG, et al. Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. *Blood*. (2011) 117:3243–6. doi: 10.1182/blood-2010-08-300384
8. Buckley RH, Schiff SE, Schiff RI, Markert L, Williams LW, Roberts JL, et al. Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med*. (1999) 340:508–16. doi: 10.1056/NEJM199902183400703
9. Chan K, Puck JM. Development of a population-based screening for severe combined immunodeficiency. *J Allergy Clin Immunol.* (2005) 115:391–8. doi: 10.1016/j.jaci.2004.10.012
10. 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. *Clin Immunol*. (2019) 202:33–9. doi: 10.1016/j.clim.2019.03.012
11. King J, Hammarström L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol*. (2018) 38:56–66. doi: 10.1007/s10875-017-0455-x
12. Routes J, Verbsky J. Newborn screening for severe combined immunodeficiency. curr allergy *Asthma Rep*. (2018) 18:34. doi: 10.1007/s11882-018-0783-9
13. Speckmann C, Neumann C, Borte S, la Marca G, Sass JO, Wiech E, et al. Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. *J Allergy Clin Immunol*. (2012) 130:991–4. doi: 10.1016/j.jaci.2012.04.004
14. Borte S, Wang N, Oskarsdóttir S, von Döbeln U, Hammarström L. Newborn screening for primary immunodeficiencies: beyond SCID and XLA. *Ann N Y Acad Sci*. (2011) 1246:118–30. doi: 10.1111/j.1749-6632.2011.06350.x
15. Puck JM. Laboratory technology for population-based screening for severe combined immunodeficiency in neonates: the winner is T-cell receptor excision circles. *J Allergy Clin Immunol*. (2012) 129:607–16. doi: 10.1016/j.jaci.2012.01.032
16. Modell V, Knaus M, Modell F. An analysis and decision tool to measure cost benefit of newborn screening for severe combined immunodeficiency (SCID) and related T-cell lymphopenia. *Immunol Res*. (2014) 60:145–52. doi: 10.1007/s12026-014-8485-4
17. Ding Y, Thompson JD, Kobrynski L, Ojodu J, Zarbalian G, Grosse SD. Cost-effectiveness/cost-benefit analysis of newborn screening for severe combined immune deficiency in Washington State. *J Pediatr.* (2016) 172:127–35. doi: 10.1016/j.jpeds.2016.01.029
18. 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-years pilot TREC and KREC screening study. *J Clin Immunol*. (2017) 37:51–60. doi: 10.1007/s10875-016-0347-5
19. Van der Ploeg CPB, Blom M, Bredius RGM, van der Burg M, Schielen PCJI, Verkerk PH, et al. Cost-effectiveness of newborn screening for severe combined immunodeficiency. *Eur J Pediatr*. (2019) 178:721. doi: 10.1007/s00431-019-03346-3
20. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, et al. International Union of Immunological Societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *J Clin Immunol.* (2018) 38:96–128. doi: 10.1007/s10875-017-0464-9
21. Dieli-Crimi R, Martínez-Gallo M, Franco-Jarava C, Antolin M, Blasco L, Paramonov I, et al. Th1-skewed profile and excessive production of proinflammatory cytokines in a NFKB1-deficient patient with CVID and severe gastrointestinal manifestations. *Clin Immunol.* (2018) 195:49–58. doi: 10.1016/j.clim.2018.07.015
22. 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. doi: 10.1016/j.clim.2017.05.007
23. 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) 34:323–30. doi: 10.1007/s10875-014-0007-6
24. van der Spek J, Groenwold RH, van der Burg M, van Montfrans JM. TREC based newborn screening for severe combined immunodeficiency disease: a systematic review. *J Clin Immunol.* (2015) 35:416–30. doi: 10.1007/s10875-015-0152-6
25. 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) 88:e12699. doi: 10.1111/sji.12699

26. 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) 27:70–7. doi: 10.1111/pai.12501
27. Borte S, Puck J, Lorey F, McGhee SA, Hershfield MS, Fasth A, et al. KRECs but not TRECs identify patients with delayed-onset adenosine deaminase (ADA) deficiency in neonatal screening. *J Clin Immunol.* (2013) 33:673.
28. Routes JM, Grossman WJ, Verbsky J, Laessig RH, Hoffman GL, Brokopp CD, et al. Statewide newborn screening for severe T-cell lymphopenia. *JAMA*. (2009) 302:2465–70. doi: 10.1001/jama.2009.1806
29. 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) 34:289–303. doi: 10.1007/s10875-014-0006-7
30. 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) 32:82–8. doi: 10.1007/s10875-011-9609-4
31. 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:e20182300. doi: 10.1542/peds.2018-2300

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Dilemma of Reporting Incidental Findings in Newborn Screening Programs for SCID: Parents' Perspective on Ataxia Telangiectasia

*Maartje Blom[1], Michiel H. D. Schoenaker[2], Myrthe Hulst[3], Martine C. de Vries[4], Corry M. R. Weemaes[5], Michèl A. A. P. Willemsen[2,5], Lidewij Henneman[6] and Mirjam van der Burg[1]**

[1] Department of Pediatrics, Leiden University Medical Centre, Leiden, Netherlands, [2] Department of Pediatric Neurology, Radboudumc Amalia Children's Hospital and Donders Institute for Brain, Cognition and Behavior, Radboud University Medical Center, Nijmegen, Netherlands, [3] Department of Biologicals, Innovation and Screening, National Institute for Public Health and the Environment, Bilthoven, Netherlands, [4] Department of Medical Ethics and Health Law, Leiden University Medical Center, Leiden, Netherlands, [5] Department of Pediatrics, Radboudumc Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, Netherlands, [6] Department of Clinical Genetics, Amsterdam Reproduction & Development Research Institute, Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam, Netherlands

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Jolan Eszter Walter, University of South Florida, United States

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***Correspondence:**
Mirjam van der Burg
m.van_der_burg@lumc.nl

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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 (NBS) 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 vs. disadvantages of early and late diagnosis of A-T.

Methods: A questionnaire was developed and sent to 4,000 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 4,000 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 asymptomatic "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.

Keywords: ataxia telangiectasia, A-T, newborn screening, severe combined immunodeficiency, SCID, incidental finding, parents' perspective, questionnaire

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 (1, 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 $n = 5$ (3), France $n = 1$ (6), Sweden $n = 1$ (5)].

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 toward 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 8–10 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 5 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 ∼20 min to complete.

The scenarios included two cases of children with A-T: one with a late diagnosis of A-T at the age of 4 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. (17) 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).

TABLE 1 | Sociodemographics of the respondents.

Variables	Research population *N* = 659	Reference group Dutch population *N* (x1000)	*p*-value
Age in years (SD)		Dutch parents[a]	
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		

Missing values were excluded from the percentages.
[a]*Reference population Dutch Parents (18). One sample t-test.*
[b]*Reference population Dutch population age 20–50 years (19).* χ^2 *test.*
[c]*Reference population Dutch population age 20–50 years (19).* χ^2 *test.*
[d]*Reference population Dutch population households (20).* χ^2 *test.*
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 (21). χ^2 *test.*
[f]*Reference population Dutch parents (20).* χ^2 *test for trend.*

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 (see **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 toward 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.

RESULTS

Response and Demographics

A total of 664 of 4,000 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 (19, 21). The average number of children was 1.73 (range 1–11 children) compared to 1.61 in the reference population of Dutch parents (20) (**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]Answers included a broad spectrum of disorders such as malignancies, diabetes mellitus, cardiovascular diseases, and autoimmune diseases.

Attitude Toward 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%)

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.

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.

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

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

Survey question:	Level of agreement[a] (%)					Ratings 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.

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

Survey question:	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]
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				Fully agree	Fully disagree				Fully agree	0.403
	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)	
	Missing 1					Missing 3					
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.	No			Yes		No			Yes		0.003
	8 (24%)			25 (76%)		49 (8.6%)			523 (91.4%)		
	Missing 2					Missing 10[d]					

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 sent to parents of A-T patients (17). [c] χ^2 test. [d]n = 77 answered "don't know" and were excluded from analysis.

(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. (17) 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 vs. 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]

TABLE 7 | Multivariate logistic correlation.

Predictor variable	A-T should be added to NBS program			Intended participation NBS A-T		
	B	SE	*p*-value	B	SE	*p*-value
Age (20–30 years)	1.001	0.617	0.105	−0.205	0.608	0.736
Gender (female)	0.409	0.488	0.402	−0.137	0.409	0.738
Ethnicity (Dutch)	−1.327	0.743	0.74	1.090	0.612	0.075
Educational level (high)	−0.11	0.382	0.977	−15.927	1929.242	0.736
Number of children (first child)	17.173	0.623	0.0001	16.097	0.653	0.0001
Having a sick child (yes)	0.480	0.786	0.374	0.138	0.781	0.860
Having a family member with a hereditary disease (yes)	−15.998	5102,717	0.997	16.322	5405.408	0.998

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.

were not significantly associated with any of the outcome variables (**Table 7**).

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 toward 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 (17). 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 toward 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 (17). 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 (22).

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 (23). Our findings are similar to studies about NBS for other (previously considered as) untreatable disorders. The study of Weinreich et al. (16) 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. (24) 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 (24). 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 (25). 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 (26). 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" (27). Furthermore, in the study of Hayeems et al. (28) 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 (28). 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 (17) 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 toward 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 toward 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 (29), 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 (30).

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.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, the Netherlands (MEC-2017-1146). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MBl, MS, and MBu designed the study. MBl, MV, CW, MW, and LH developed the questionnaire. MBl and MH collected and analyzed the data. MBl, MH, and MBu wrote the paper. All authors edited the paper and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02438/full#supplementary-material

REFERENCES

1. 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) 312:729–38. doi: 10.1001/jama.2014.9132
2. 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. doi: 10.3389/fimmu.2017.01448
3. 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:e20182300. doi: 10.1542/peds.2018-2300
4. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol.* (2005) 115:391–8. doi: 10.1016/j.jaci.2004.10.012
5. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterstrom 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) 37:51–60. doi: 10.1007/s10875-016-0347-5
6. Thomas C, Durand-Zaleski I, Frenkiel J, Mirallie S, Leger A, Cheillan D, et al. Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. *Clin Immunol.* (2019) 202:33–9. doi: 10.1016/j.clim.2019.03.012
7. Dorsey M, Puck J. Newborn screening for severe combined immunodeficiency in the US: current status and approach to management. *Int J Neonatal Screen.* (2017) 3:15. doi: 10.3390/ijns3020015
8. Puck JM. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. *Immunol Rev.* (2019) 287:241–52. doi: 10.1111/imr.12729
9. Rothblum-Oviatt C, Wright J, Lefton-Greif MA, McGrath-Morrow SA, Crawford TO, Lederman HM. Ataxia telangiectasia: a review. *Orphan J Rare Dis.* (2016) 11:159. doi: 10.1186/s13023-016-0543-7
10. van Os NJH, Haaxma CA, van der Flier M, Merkus P, van Deuren M, de Groot IJM, et al. Ataxia-telangiectasia: recommendations for multidisciplinary treatment. *Dev Med Child Neurol.* (2017) 59:680–9. doi: 10.1111/dmcn.13424
11. van Os NJ, Roeleveld N, Weemaes CM, Jongmans MC, Janssens GO, Taylor AM, et al. Health risks for ataxia-telangiectasia mutated heterozygotes: a systematic review, meta-analysis and evidence-based guideline. *Clin Genet.* (2016) 90:105–17. doi: 10.1111/cge.12710
12. Weigelt B, Bi R, Kumar R, Blecua P, Mandelker DL, Geyer FC, et al. The landscape of somatic genetic alterations in breast cancers from ATM germline mutation carriers. *J Natl Cancer Inst.* (2018) 110:1030–4. doi: 10.1093/jnci/djy028
13. Mallott J, Kwan A, Church J, Gonzalez-Espinosa D, Lorey F, Tang LF, et al. Newborn screening for SCID identifies patients with ataxia telangiectasia. *J Clin Immunol.* (2013) 33:540–9. doi: 10.1007/s10875-012-9846-1
14. Borte S, von Dobeln 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
15. Health Council of the Netherlands. *Neonatal Screening*: *New Recommendations.* The Hague: Health Council of the Netherlands (2015).
16. Weinreich SS, Rigter T, van El CG, Dondorp WJ, Kostense PJ, van der Ploeg AT, et al. Public support for neonatal screening for Pompe disease, a broad-phenotype condition. *Orphanet J Rare Dis.* (2012) 7:15. doi: 10.1186/1750-1172-7-15
17. Schoenaker MHD, Blom M, de Vries MC, Weemaes CM, van der Burg M, Willemsen MAAP. Early diagnosis of Ataxia Telangiectasia in the neonatal phase: a parents' perspective (2019).
18. Statistics Netherlands. *Birth; Key Figures*. Statline (2017). Available online at: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37422eng/table?ts=1564486871296 (accessed 15 July, 2019).
19. Statistics Netherlands. *Population; Key Figures. Statline.* (2018). Available online at: https://opendata.cbs.nl/statline/#/CBS/en/dataset/37296eng/table?ts=1564487099871 (accessed July 15, 2019).
20. Statistics Netherlands. *Households; Size, Composition, Position in the Household,* Statline (2018). Available online at: https://opendata.cbs.nl/statline/#/CBS/en/dataset/82905ENG/table?ts=1564487413252 (accessed July 15, 2019).
21. Statistics Netherlands. *Labour Force; Level of Education by Personal Characteristics*. Statline(2019). Available online at: https://opendata.cbs.

nl/statline/#/CBS/en/dataset/71822eng/table?fromstatweb (accessed July 15, 2019).
22. Wiklund I, Wiklund J, Pettersson V, Bostrom AM. New parents' experience of information and sense of security related to postnatal care: a systematic review. *Sex Reprod Healthc.* (2018) 17:35–42. doi: 10.1016/j.srhc.2018.06.001
23. Levy PA. An overview of newborn screening. *J Dev Behav Pediatr.* (2010) 31:622–31. doi: 10.1097/DBP.0b013e3181eedf01
24. Wood MF, Hughes SC, Hache LP, Naylor EW, Abdel-Hamid HZ, Barmada MM, et al. Parental attitudes toward newborn screening for Duchenne/Becker muscular dystrophy and spinal muscular atrophy. *Muscle Nerve.* (2014) 49:822–8. doi: 10.1002/mus.24100
25. Boardman FK, Sadler C, Young PJ. Newborn genetic screening for spinal muscular atrophy in the UK: the views of the general population. *Mol Genet Genomic Med.* (2018) 6:99–108. doi: 10.1002/mgg3.353
26. Health Council of the Netherlands. *Neonatal Screening for Spinal Muscular Atrophy.* The Hague: Health Council of the Netherlands (2019).
27. Hasegawa LE, Fergus KA, Ojeda N, Au SM. Parental attitudes toward ethical and social issues surrounding the expansion of newborn screening using new technologies. *Public Health Genomics.* (2011) 14:298–306. doi: 10.1159/000314644
28. Hayeems RZ, Miller FA, Bombard Y, Avard D, Carroll J, Wilson B, et al. Expectations and values about expanded newborn screening: a public engagement study. *Health Expect.* (2015) 18:419–29. doi: 10.1111/hex.12047
29. Morton SMB, Bandara DK, Robinson EM, Carr PEA. In the 21st Century, what is an acceptable response rate? *Aust N Z J Public Health.* (2012) **36**:106–8. doi: 10.1111/j.1753-6405.2012.00854.x
30. Nakash RA, Hutton JL, Jorstad-Stein EC, Gates S, Lamb SE. Maximising response to postal questionnaires–a systematic review of randomised trials in health research. *BMC Med Res Methodol.* (2006) 6:5. doi: 10.1186/1471-2288-6-5

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Ataxia Telangiectasia Diagnosed on Newborn Screening–Case Cohort of 5 Years' Experience

*Amarilla B. Mandola[1,2], Brenda Reid[1,2], Raga Sirror[3], Rae Brager[4], Peter Dent[4], Pranesh Chakroborty[5], Dennis E. Bulman[5] and Chaim M. Roifman[1,2]**

[1] Division of Immunology and Allergy, Department of Paediatrics, The Hospital for Sick Children and the University of Toronto, Toronto, ON, Canada, [2] The Canadian Centre for Primary Immunodeficiency and the Jeffrey Modell Research Laboratory for the Diagnosis of Primary Immunodeficiency, the Hospital for Sick Children, Toronto, ON, Canada, [3] Paediatric Allergy/Immunology, Thunder Bay Regional Health Sciences Center, North Ontario School of Medicine, Thunder Bay, ON, Canada, [4] Division of Rheumatology, Immunology, and Allergy, Department of Paediatrics, McMaster Children's Hospital, McMaster University, Hamilton, ON, Canada, [5] Department of Pediatrics, CHEO Research Institute and Newborn Screening Ontario, University of Ottawa, Ottawa, ON, Canada

Ataxia telangiectasia (AT) is a genetic condition caused by mutations involving *ATM* (Ataxia Telangiectasia Mutated). This gene is responsible for the expression of a DNA double stranded break repair kinase, the ATM protein kinase. The syndrome encompasses combined immunodeficiency and various degrees of neurological abnormalities and increased risk of malignancy. Typically, patients present early in life with delay in neurological milestones, but very infrequently, with life threatening infections typical of a profound T cell deficiency. It would therefore be unexpected to identify this condition immediately after birth using T cell receptor excision circle (TREC)-based newborn screening (NBS) for SCID. We sought to evaluate the frequency of AT detected by NBS, and to assess immunity as well as the genetic aberrations associated with this early presentation. Here, we describe the clinical, laboratory, and genetic features of patients diagnosed with AT through the Ontario NBS program for SCID, and followed in our center since its inception in 2013. Four patients were diagnosed with AT as a result of low TRECs on NBS. In each case, whole exome sequencing was diagnostic. All of our patients had compound heterozygous mutations involving the FRAP-ATM-TRRAP (FAT) domain of the *ATM* gene, which appears critical for kinase activity and is highly sensitive to mutagenesis. Our patients presented with profound lymphopenia involving both B and T cells. The ratio of naïve/memory CD45+RA/RO T cells population was variable. T cell repertoire showed decreased T cell diversity. Two out of four patients had decreased specific antibody response to vaccination and hypogammaglobulinemia requiring IVIG replacement. In two patients, profound decreased responses to phytohemagglutinin stimulation was observed. In the other two patients, the initial robust response declined with time. In summary, the rate of detection of AT through NBS had been surprisingly high at our center. One case was identified per year, while the total rate for SCID has been five new cases per year. This early detection may allow for better prospective evaluation of AT shortly after birth, and may assist in formulating early and more effective interventions both for the neurological as well as the immune abnormalities in this syndrome.

Keywords: ataxia telangiectasia, newborn screen, primary immunodeficiency, TRECs, lymphopenia

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***Correspondence:**
Chaim M. Roifman
chaim.roifman@sickkids.ca

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INTRODUCTION

The protein kinase Ataxia Telangiectasia Mutated (ATM) is a high molecular weight PI3K-family kinase involved in the phosphorylation of multiple proteins, including in key cellular functions such as gene transcription and expression, response to oxidative stress, and energy metabolism. Together, ATM acts as a temporal gate keeper for proper cell division and appropriate repair (1–3).

Ataxia telangiectasia (AT) is an autosomal recessive syndrome encompassing progressive neuronal degeneration, ocular and cutaneous telangiectases, variable immunodeficiency, and cancer susceptibility. The ESID diagnostic criteria for AT includes ataxia and at least two of the following features: (a) oculo-cutaneous telangiectasia, (b) elevated alpha-fetoprotein (AFP), (c) typical AT karyotype (translocation of chromosomes 7; 14), (d) cerebellum hypoplasia on MRI (4). The criteria do not include neurological abnormalities, likely because of its inconsistency, great variability, and due to challenges in obtaining data from pediatric examination.

By controlling cell cycle and DNA repair, ATM plays an important role in the development and function of both the cellular and humoral immune system. The development of appropriate T cell receptor (TCR) repertoire is dependent on the repair of V(D)J recombination-induced breaks by the non-homologous end-joining (NHEJ) pathway, which is promoted by ATM (5, 6). Proper class-switch recombination is facilitated by ATM, through the correction of breaks by a Ku-dependent end-joining pathway, as well as preventing aberrant translocations due to double strand breaks and propagating the Ku-independent alternative NHEJ (A-NHEJ) pathway (5, 7, 8).

Diagnosis of AT patients may be delayed due to the wide variability in clinical phenotype; the syndrome is frequently confused with cerebral palsy and the immunological evaluation overlooked or misdiagnosed as Hyper-immunoglobulin M syndrome (9–12).

Review of the literature reveals that the immunological presentation of AT is highly variable; IgA concentrations are low in more than 50% of cases, IgM levels are elevated in up to 60% of cases, but low IgG is infrequent (occurring in 10–18% of cases). Numbers of T cells, in particular CD4+ cells, may decline over time in 30–75% of cases. However, profound lymphopenia in neonates was not well-recognized prior to the introduction of NBS (13–15), and the AT cases that have since been identified by TREC/KREC in infants (16–18) are likely an underestimate of the true number of patients affected. The complete absence of ATM enzyme activity is much more likely to result in clinical and/or immunological features of immunodeficiency compared to those who retain residual activity (19).

We describe here four patients with AT identified by the NBS system in Ontario, Canada.

METHODS

Patients

This study conformed to the Declaration of Helsinki and all local ethical requirements. Information on presentation, complications, laboratory parameters, management, and outcomes were compiled both prospectively and retrospectively using parent interview and medical chart review. All laboratory results were analyzed with reference to age-related normal ranges. Written informed consent was obtained from the parents or guardians of the participant for the publication of this study.

TREC Determination From Guthrie Blood Spots

Methodology for the assessment of TREC levels by qPCR was performed as previously described (20).

Lymphocyte Proliferation

Lymphocyte proliferative responses were assayed to mitogens including phytohemagglutinin (PHA) and anti-CD3. All assays were performed in triplicate and were compared with simultaneously stimulated normal controls, as previously described (21).

Chromosome Breakage Analysis and G-Band Evaluation

For chromosome spontaneous breakage frequency evaluation, fifty metaphases were examined by solid stain analysis. One hundred metaphase cells from a 3-day PHA-stimulated lymphocyte culture were examined by G-band analysis. Chromosome rearrangements involving regions 7p14, 7q34, and 14q11.2 were evaluated in metaphase cells.

Whole Exome Sequencing and Variant Calling

DNA from blood was submitted to The Center for Applied Genomics (TCAG), Toronto, Canada for exome library preparation and sequencing. DNA was quantified by Qubit DNA HS assay (Life Technologies, Carlsbad, CA) and 100 ng of input DNA was used for library preparation using the Ion AmpliSeq Exome Kit (Life Technologies) according to the manufacturer's recommendations. The Ampliseq Exome library was immobilized on Ion PI™ Ion Sphere™ particles using the Ion PI Template OT2 200 Kit v3. Sequencing was performed with the Ion PI Sequencing 200 Kit v3 and Ion PI Chip v2 in the Ion Proton™ semiconductor sequencing system following the manufacturer's recommendation. Alignment and variant calling were performed using Torrent Suite (v4.0) on the Ion Proton Server, using the Ion Proton AmpliSeq germline low stringency setting and the hg19 reference genome. The variants were annotated using an in-house annotation pipeline (22) based on Annovar (November 2014 version) (23) and RefSeq gene models (downloaded from UCSC 01 August 2015).

Sanger Sequencing

Patients' genomic DNA was extracted from peripheral blood lymphocytes using the Geneaid Genomic DNA Mini Kit. Genomic DNA was amplified by PCR with specific primers designed upstream and downstream of the ATM gene. Sequencing was done using GenomeLab Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman

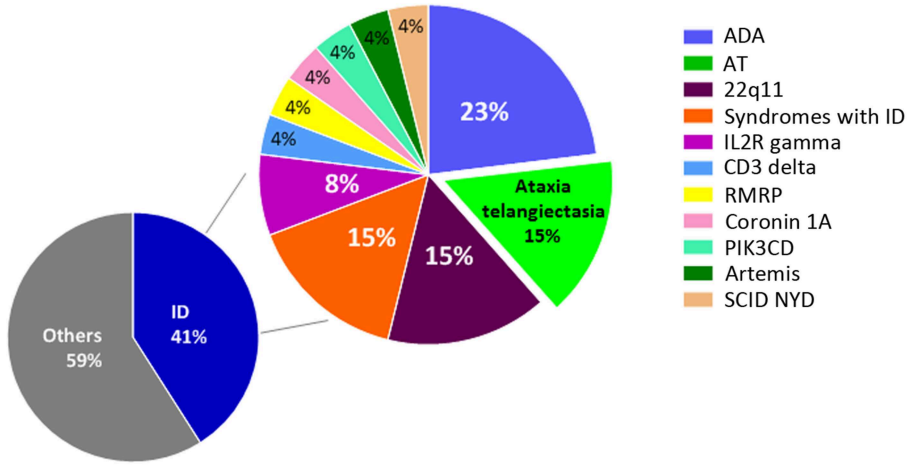

FIGURE 1 | Positive NBS results for SCID at the Hospital for Sick Children from 2013 to 2018. A total of 63 infants were NBS positive for SCID at our center. Of the 26 cases with confirmed immunodeficiency, 23% were diagnosed with adenosine deaminase deficiency (ADA), 15% with ataxia telangiectasia (AT), 15% with syndromes involving immunodeficiency (ID), 8% with IL2R gamma deficiency, and 4% each with deficiencies of CD3 delta, RMRP, coronin 1A, PIK3CD, Artemis, and SCID not yet defined (NYD). The remaining 37 cases were associated with maternal immunosuppression, cardiac post-thymectomy, prematurity, and lymphopenia NYD.

Coulter) and analyzed on CEQ 8000 Genetic Analysis System (Beckman Coulter).

RESULTS

NBS for Severe Combined Immunodeficiency (SCID) was first introduced in Ontario, Canada, in 2013, and has since been expanded to the Maritime provinces of New Brunswick, Nova Scotia, and Prince Edward Island in 2016. This lifesaving test has yet to be introduced in other jurisdictions of Canada. In Ontario, where there are ∼140,000 births/year, an unexpectedly high number of patients with AT were diagnosed at our center. The Hospital for Sick Children, Toronto, Ontario, is a quaternary hospital with a catchment area of 10 million people. Over the past 5 years, 63 infants at our center were screened positive for SCID on NBS for various reasons (**Figure 1**). At this point we can conclude that primary immunodeficiencies are more common than estimated in the past, and recent reports suggest that around 1% of the global population may be affected (4, 24–26). The prevalence of AT is estimated to be between 1 in 40,000 and 1 in 100,000 live births, though in certain populations the frequency of mutations are different due to founder effects (14, 27).

Patients

Patient 1

Patient 1 is the second-born child of a non-consanguineous union with parents of Eastern European origins, with unremarkable family history except for diabetes in a paternal uncle (**Figure 2A**). His first physical examination at 3 weeks of age was unremarkable, with normal head circumference (30th percentile). At age 12 months, he developed a wobbly gait with mild hypotonia and at 18 months he had oculomotor apraxia, excessive drooling indicative of oro-motor apraxia, and appendicular hypotonia. He had slower baseline gait than expected for age, and truncal ataxia was prominent when walking. Delayed motor development was also observed. His brain MRI was normal. Lymphocyte immunophenotyping revealed reduced numbers of CD19+ B cells, CD3+, CD4+, and CD8+ T cells, which further declined at 8 months of age (**Table 1**). The number of CD4+ naïve T cells were diminished, suggesting ineffective thymic production or egress of these cells (**Table 2**). His T cell repertoire showed decreased diversity, while lymphocyte proliferation responses to PHA were preserved. His humoral evaluation at that time showed normal age referenced immunoglobulin levels, however, the patient's family decided against vaccination, and thus specific antibody titers were not assessed. His AFP level was slightly above normal.

Patient 2

Patient 2 is the third child born to a non-consanguineous family of English extraction, with a family history of breast cancer in the maternal grandmother and paternal aunt. In addition, the paternal grandmother was diagnosed with cervical cancer and a paternal uncle with a bone tumor (**Figure 2B**). His first physical examination at age 4 weeks was remarkable for mild axial hypotonia which continued to progress to wobbly gait, stagger, ataxia and mild diffuse hypotonia which were detected at 10 months. The patient's initial immune evaluation as a newborn revealed lymphopenia including reduced numbers of CD19+ B cells, CD3+, CD4+, and CD8+ T cells (**Table 1**). A repeat evaluation at age 8 months showed progressive lymphopenia with a reduction in CD4+ naïve T cells as well as reduced T cell repertoire diversity (**Table 2**). Lymphocyte responses to PHA were decreased. His immunoglobulin levels at both 8 and 18 months of age were normal, with sustained specific antibody titres to diphtheria and tetanus.

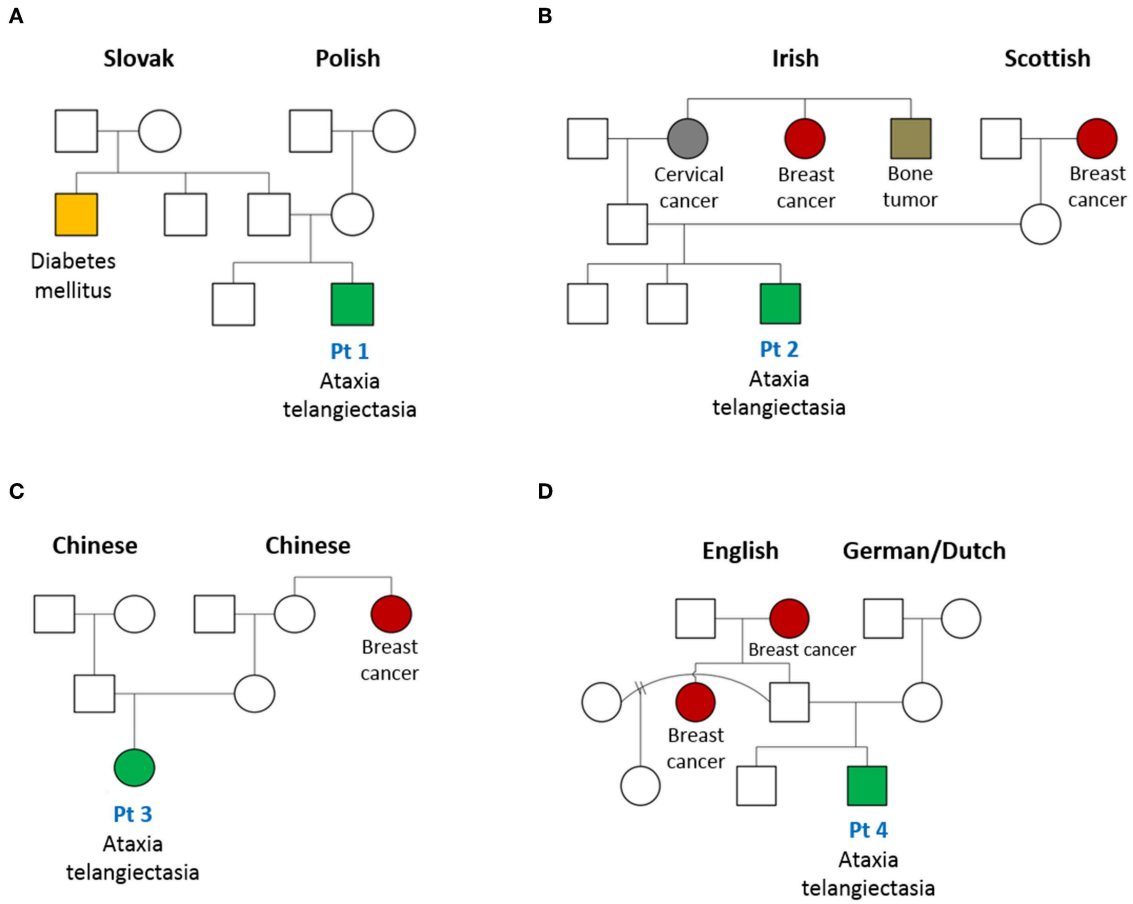

FIGURE 2 | Pedigree of patients with AT diagnosed on newborn screen for SCID. Family tree of patients (Pt) 1–4 (**A–D**, respectively) in this case series are shown. An increased frequency of cancer, especially breast cancer, in female family members is noted.

TABLE 1 | Initial immune evaluation at SCID retrieval.

	Pt 1	Pt 2	Pt 3	Pt 4	Reference range
White blood cell count	6.4	3.05	5.4	5.7	5–20 × 10^9cells/μL
Neutrophil count	2.6	0.55	2.21	2.91	1–9.5 × 10^9cells/μL
Lymphocyte count	**1.8**	**1.52**	**1.73**	**1.22**	2–17 × 10^9cells/μL
Eosinophil count	0.8	0.25	0.22	0.83	0.07–1 × 10^9cells/μL
Thrombocyte count	440	323	547	**737**	150–400 × 10^9cells/μL
CD3+	**888**	**780**	**1142**	**664**	2,300–6,500 cells/μL
CD19+	**234**	**79**	**43**	**154**	600–3,000 cells/μL
CD3+/CD4+	**633**	**262**	**349**	**421**	1,500–5,000 cells/μL
CD3+/CD8+	**233**	**485**	759	**228**	500–1,600 cells/μL
NK	528	531	440	258	100–1,300 cells/μL
PHA (Stimulation Index, SI)	563	**416**	**151**	**158**	>450 SI
CD3 Mitogen Stimulation	ND	Normal	**7.2 (C:216)**	**9.3 (C:255)**	

C, control; ND, not determined; bold text indicates values that fall outside of the reference range.

Patient 3

Patient 3 is the first-born child of a non-consanguineous family of Chinese ethnicity, with a family history of breast cancer in the maternal aunt (**Figure 2C**). Her first physical examination at age 3 weeks revealed axial hypotonia and her head circumference was below the 5th percentile for age, where it remained thereafter. At 12 months of age, profound hypotonia, nystagmus, unbalanced gait, and feeding problems became apparent, and

TABLE 2 | Immunological evaluation at age 8 months.

	Pt 1	Pt 2	Pt 3	Pt 4	Reference range
White blood cell count	6.18	5.13	3.2	3.4	5–20 × 10^9cells/μL
Neutrophil count	2.8	2.9	1.09	0.8	1–9.5 × 10^9cells/μL
Lymphocyte count	**1.7**	**1.27**	**0.34**	**1.5**	2–17 × 10^9cells/μL
Eosinophil count	0.38	0.1	0.26	0.4	0.07–1 × 10^9cells/μL
Thrombocyte count	510	394	466	614	150–400 × 10^9cells/μL
CD3+	**645**	**677**	**664**	**823**	2,300–6,500 cells/μL
CD19+	**118**	**186**	**116**	**201**	600–3,000 cells/μL
CD3+/CD4+	**466**	**370**	**441**	**550**	1,500–5,000 cells/μL
CD3+/CD8+	**109**	**242**	**182**	**235**	500–1,600 cells/μL
NK	685	531	581	547	100–1,300 cells/μL
CD4+/CD45+ RA+	**10 (C:44)**	**16.4 (C:34)**	**11 (C:33)**	**14 (C:23)**	
CD4+/CD45+ RO+	38 (C:17)	39.8 (C:19)	10 (C:12)	20 (C:9)	
IgG	2.8	5.5	**<1.1**	**<1.1**	1.1–7.0 g/L
IgM	0.7	0.2	**1**	**1.2**	0.1–0.7 g/L
IgA	0.1	0.1	**<0.1**	**<0.1**	0.1–3 g/L
Anti-pneumococcal Ab	Not vaccinated	11	on IVIG	on IVIG	
Anti-tetanus toxoid Ab	Not vaccinated	2.86	0.16	0.03 (5 mo)	>0.1
Diphtheria toxoid Ab	Not vaccinated	3	on IVIG	on IVIG	>0.01
AFP	**23**	**56**	**152**	**70**	<21 ng/mL

Ab, antibody; C, control; IVIG, intravenous immunoglobulin; mo, months; bold text indicates values that fall outside of the reference range.

at 18 months she was found to have additional neurological features including dysarthria, dysmetria, nystagmus, ataxia, and oculo-oro-motor apraxia. The patient's initial immune evaluation while a newborn demonstrated reduced numbers of CD19+ B cells, CD3+, CD4+, and CD8+ T cells which further declined when assessed at 8 months of age (**Table 1**). The number of CD4+ naïve T cells were reduced and she had a limited T cell repertoire (**Table 2**). Her lymphocyte proliferation responses to PHA were remarkably depressed. Humoral evaluation at age 8 months showed severe hypogammaglobulinemia including low IgG (IgG < 1.1), low IgA level (IgA < 0.1), elevated IgM (IgM = 1), and undetectable antibody responses to vaccines, requiring immunoglobulin replacement. Her AFP at age 8 months was greatly elevated (152).

Patient 4

Patient 4 is the second-born child of a non-consanguineous family of European origin, with a family history of breast cancer in a paternal aunt and grandmother (**Figure 2D**). His initial physical examination at age 3 weeks was unremarkable, but at 12 months he developed a wobbly gait with mild hypotonia, and at 18 months he had ataxia with unstable stance and gait, drooling, and oro-motor apraxia. The patient's initial immune evaluation while a newborn revealed reduced numbers of CD19+ B cells, CD3+, CD4+, and CD8+ T cells (**Table 1**), with further decline observed at 8 months (**Table 2**). His T cell repertoire was limited, and lymphocyte proliferation responses to PHA were very low. He had severe hypogammaglobulinemia including low IgG (IgG < 1.1) and low IgA level (<0.1), elevated IgM (1.2), and undetectable titers to childhood vaccines, requiring immunoglobulin replacement. His AFP at age 8 months was very elevated (70 ng/mL).

Genetic Work Up

Following the finding of a positive NBS for SCID, and in the context of broader presenting features, we performed G-band analysis that, in each of these patients, demonstrated significant chromosome re-arrangements involving T cell receptor gamma, beta, and alpha/delta gene loci at chromosome locations 7p14, 7q35, and 14q11.2, and elevated frequency of spontaneous breakage. Whole exome sequencing (WES) revealed compound heterozygous mutations in the *ATM* gene in each patient (**Table 3**). The mutations were confirmed by Sanger sequencing and segregation studies showed that parents were heterozygous carriers of those mutations.

In Patient 1, WES revealed a c.331+1G>A mutation predicting p.Ser111Asn amino acid change affecting a splice donor site, and possibly disrupting the HEAT (Huntingin, elongation factor 3, protein phosphatase 2A, TOR1) domain. The second mutation, c.6095G>A, predicting p.Arg2032Lys amino acid change involves the FAT (Focal adhesion kinase targeting) domain. In Patient 2, two pathogenic variants, c.170G>A (p.Trp57*) and c.6997dupA (p.Thr2333Asnfs*40), involving both the HEAT and FAT domains were identified. Genetic evaluation of Patient 3 revealed two mutations within the FAT domain, c.6679C>T, (p.Arg2227Cys; pathogenic), c.7090-1G>A (p.Lys2363Arg; novel). Similarly, in Patient 4, the mutations c.5228C>T (p.Thr1743Ile; likely pathogenic) and c.6908dupA

TABLE 3 | SCID NBS TREC levels and genetic evaluation results.

	Pt 1	Pt 2	Pt 3	Pt 4
TRECs (copies/ 3 μL DNA) (cut-off >75 copies/3 μL)	**22**	**23**	**26**	**41**
WES/Sanger sequencing	c.331+1G>A; c.6095G>A	c.170G>A c.6997dupA	c.6679C>T c.7090-1G>A	c.5228C>T c.6908dupA
Affected region	FAT domain HEAT repeats	FAT domain HEAT repeats	FAT domain FAT domain	FAT domain FAT domain
G-band analysis assay	**Positive**	**Positive**	**Positive**	**Positive**

Bold text indicates values that fall outside of the reference range.

(p.Glu2304Glyfs*69; pathogenic) were both localized to the FAT domain.

DISCUSSION

The implementation of TREC-based SCID NBS in Ontario, Canada, has enabled the early detection and diagnosis of SCID that would otherwise be missed or delayed until the onset of life-threatening infections. Unfortunately, it appears that many cases of significant T cell deficiencies cannot be detected by this methodology. Surprisingly, some non-SCID conditions have been rarely detected by NBS (28). AT has not been typically regarded as having a SCID-like clinical course or fate.

In our cohort, we describe four patients with AT who all presented with low TRECs on SCID NBS. The initial approach to patients with an abnormal SCID NBS in Canada is described in Biggs et al. (29). All had profound, sustained B and T cell lymphopenia, which is consistent with low thymic output. Our patients had low naïve CD4+/CD45+ RA+ populations compared to age appropriate controls. Three patients presented with decreased lymphocyte proliferation responses. Two out of the four patients showed early onset humoral immunodeficiency and were started on immunoglobulin replacement therapy.

Patients with AT are rarely diagnosed in the first year of life, largely because their typical neurological manifestations are noted at a later age. Many are incorrectly diagnosed with cerebral palsy. Early detection at the newborn age leads to the correct diagnosis and might aid in early interventions. However, this may pose an ethical conundrum since some jurisdictions, such as the Netherlands, do not allow the screening and reporting of diseases for which there is no cure. In Ontario, the finding of a positive SCID newborn screen, regardless of underlying cause, triggers urgent follow-up evaluation in accordance with our Ministry of Health-approved algorithm for assessment and treatment of such cases (30).

Each of our patients carry compound heterozygous mutations in the *ATM* gene, one of which is a pathogenic variant localized to the FAT domain. As expected, patients' pedigrees also show an increased frequency of cancer, especially breast cancer in female family members.

The detection of AT by NBS was first reported in 2012 in Swedish newborns (6) and subsequently in other jurisdictions where NBS for SCID has been implemented (31, 32). We have shown here that AT, if detected early by TREC-based NBS, has a more profound immunological and neurological phenotype, and this intuitively might predict a more severe disease course.

Early genetic diagnosis of AT enables very early patient centered and individualized interventions, including physiotherapy, neurological support, proper immunological evaluation, and infection prevention before onset of complications (PJP prophylaxis and IVIG before the development of bronchiectasis). Moreover, it may also aid in further understanding possible genotype-phenotype correlations.

CONCLUSION

We have demonstrated that AT can be detected shortly after birth by NBS for SCID. AT should be considered in cases with NBS positivity because of its relatively high frequency, as shown in our cohort (15%). This early detection allows for early referral to specialized centers for comprehensive evaluation and guidance. It enables the provision of individualized intervention for patients as well as genetic counseling for the family members, especially mothers, who, as carriers, have an increased risk of developing breast cancer and other malignancies. Furthermore, the early detection of immune abnormalities allows for appropriate treatment to prevent or minimize the otherwise complicated disease course that AT patients may suffer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this study will be made available upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Board, Hospital for Sick Children. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CR and AM conceptualized and designed the study and wrote the initial draft of the manuscript. BR, RS, RB, and PD were involved in the clinical care of the patients. DB and PC evaluated NBS TRECs. All authors contributed to manuscript revision, read, and approved the submitted manuscript.

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REFERENCES

1. McKinnon PJ. ATM and the molecular pathogenesis of ataxia telangiectasia. *Ann Rev Pathol.* (2012) 7:303–21. doi: 10.1146/annurev-pathol-011811-132509
2. Ambrose M, Gatti RA. Pathogenesis of ataxia-telangiectasia: the next generation of ATM functions. *Blood.* (2013) 121:4036–45. doi: 10.1182/blood-2012-09-456897
3. Schon K, van Os NJH, Oscroft N, Baxendale H, ScoffingsD, Ray J, et al. Genotype, extrapyramidal features, and severity of variant ataxia-telangiectasia. *Ann Neurol.* (2019) 85:170–80. doi: 10.1002/ana.25394
4. Seidel MG, Kindle G, Gathman B, Quinti I, Buckland M, van Montfrans J, et al. The European Society for Immunodeficiencies (ESID) registry working definitions for the clinical diagnosis of inborn errors of immunity. *J Aller Clin Immun.* (2019) 7:1763–70. doi: 10.1016/j.jaip.2019.02.004
5. Bredemeyer AL, Sharma GG, Huang CY, Helmink BA, Walker LM, Khor KC, et al. ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature.* (2006) 442:466–70. doi: 10.1038/nature04866
6. 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
7. Lumsden JM, McCarty T, Petiniot LK, Shen R, Barlow C, Wynn TA, et al. Immunoglobulin class switch recombination is impaired in atm-deficient mice. *J Exp Med.* (2004) 200:1111–21. doi: 10.1084/jem.20041074
8. Reina-San-Martin B, Chen HT, Nussenzweig A, Nussenzweig MC. ATM is required for efficient recombination between immunoglobulin switch regions. *J Exp Med.* (2004) 200:1103–10. doi: 10.1084/jem.20041162
9. van Os NJH, Jansen AFM, van Deuren M, Haraldsson A, van Driel NTM, Etzioni A, et al. Ataxia-telangiectasia: immunodeficiency and survival. *Clin Immunol.* (2018) 178:45–55. doi: 10.1016/j.clim.2017.01.009
10. Azarsiz E, Karaca NE, Gunaydin NC, Gulez N, Ozturk C, Aksu G, et al. Do elevated serum IgM levels have to be included in probable diagnosis criteria of patients with Ataxia-telangiectasia? *Int J Immunopathol Pharmacol.* (2014) 27:421–7. doi: 10.1177/039463201402700312
11. Aghamohammadi A, Imai K, Moazzami K, Abolhassani H, Tabatabaeiyan M, Parvaneh N, et al. Ataxia-telangiectasia in a patient presenting with hyper-immunoglobulin M syndrome. *J Investig Allergol Clin Immunol.* (2010) 20:442–5.
12. Noordzij JG, Wulffraat NM, Haraldsson A, Meyts I, van't Veer LJ, Hogervorst FB, et al. Ataxia-telangiectasia patients presenting with hyper-IgM syndrome. *Arch Dis Child.* (2009) 94:448–9. doi: 10.1136/adc.2008.149351
13. Minto H, Mensah KA, Reynolds PR, Meffre E, Rubtsova K, Gelfand EW. A novel ATM mutation associated with elevated atypical lymphocyte populations, hyper-IgM, and cutaneous granulomas. *Clin Immunol.* (2019) 200:55–63. doi: 10.1016/j.clim.2019.01.002
14. Rothblum-Oviatt C, Wright J, Lefton-Greif M, McGrath-Morrow SA, Crawford TO, Lederman HM. Ataxia telangiectasia: a review. *Orphanet J Rare Dis.* (2016) 11:1–21. doi: 10.1186/s13023-016-0543-7
15. Nowak-Wegrzyn A, Crawford TO, Winkelstein JA, Carson KA, Lederman HM. Immunodeficiency and infections in ataxia-telangiectasia. *J Pediatr.* (2004) 144:505–11. doi: 10.1016/j.jpeds.2003.12.046
16. Kamae C, Nakagawa N, Sato H, Honma K, Mitsuiki N, Ohara O, et al. Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles. *J Allergy Clin Immunol.* (2013) 131:1437–1440.e5. doi: 10.1016/j.jaci.2012.10.059
17. 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) 27:70–7. doi: 10.1111/pai.12501
18. 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) 2018:e12699. doi: 10.1111/sji.12699
19. Driessen GJ, Ijspeert H, Weemaes CM, Haraldsson Á, Trip M, Warris A, et al. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed b- and t-cell homeostasis and reduced immune repertoire diversity. *J Allergy Clin Immunol.* (2013) 131:1367–75.e9. doi: 10.1016/j.jaci.2013.01.053
20. Suresh S, Dadi H, Reid B, Vong L, Bulman DE, Roifman CM. Time-dependent decline of T-cell receptor excision circle levels in ZAP-70 deficiency. *J Allergy Clin Immunol Pract.* (2019). doi: 10.1016/j.jaip.2019.08.018. [Epub ahead of print].
21. Sharfe N, Nahum A, Newell A, Dadi H, Ngan B, Pereira SL, et al. Fatal combined immunodeficiency associated with heterozygous mutation in STAT1. *J Aller Clin Immunol.* (2014) 133:807–17. doi: 10.1016/j.jaci.2013.09.032
22. Stavropoulos DJ, Merico D, Jobling R, Bowdin S, Monfared N, Thiruvahindrapuram B, et al. Whole genome sequencing expands diagnostic utility and improves clinical management in paediatric medicine. *NPJ Genom Med.* (2016) 1:15012. doi: 10.1038/npjgenmed.2015.12
23. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* (2010) 38:e164. doi: 10.1093/nar/gkq603
24. Boyle JM, Buckley RH. Population prevalence of diagnosed primary immunodeficiency diseases in the United States. *J Clini Immunol.* (2007) 27:497–502. doi: 10.1007/s10875-007-9103-1
25. Modell V, Orange JS, Quinn J, Modell F. Global report on primary immunodeficiencies: 2018 update from the jeffrey modell centers network on disease classification, regional trends, treatment modalities, and physician reported outcomes. *Immunol Res.* (2018) 66:367–80. doi: 10.1007/s12026-018-8996-5
26. Bousfiha AA, Jeddane L, Ailal F, Benhsaien I, Mahlaoui N, Casanova JL, et al. Primary immunodeficiency diseases worldwide: more common than generally thought. *J Clin Immunol.* (2013) 33:1–7. doi: 10.1007/s10875-012-9751-7
27. Teive HA, Moro A, Moscovich M, Arruda WO, Munhoz RP, Raskin S, et al. Ataxia-telangiectasia - a historical review and a proposal for a new designation: ATM syndrome. *J Neurol Sci.* (2015) 355:3–6. doi: 10.1016/j.jns.2015.05.022
28. Kwan A, Puck JM. History and current status of newborn screening for severe combined immunodeficiency. *Semin Perinatol.* (2015) 39:194–205. doi: 10.1053/j.semperi.2015.03.004
29. Biggs CM, Haddad E, Issekutz TB, Roifman CM, Turvey SE. Newborn screening for severe combined immunodeficiency: a primer for clinicians. *CMAJ.* (2017) 189:E1551–7. doi: 10.1503/cmaj.170561
30. Reid B, Ovadia A, Dinur Schejter Y. Managing newborn screening for SCID in a referral centre. *LymphoSign J.* (2017) 4:77–9. doi: 10.14785/lymphosign-2017-0005
31. Mallott J, Kwan A, Church J, Gonzalez-Espinosa D, Lorey F, Tang LF, et al. Newborn screening for SCID identifies patients with ataxia telangiectasia. *J Clin Immunol.* (2013) 33:540–9. doi: 10.1007/s10875-012-9846-1
32. Cousin MA, Smith MJ, Sigafoos AN, Jin JJ, Murphree MI, Boczek NJ, et al. Utility of DNA, RNA, protein, and functional approaches to solve cryptic immunodeficiencies. *J Clin Immunol.* (2018) 38:307–19. doi: 10.1007/s10875-018-0499-6

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Newborn Screening for Primary Immunodeficiencies: The Gaps, Challenges, and Outlook for Developing Countries

*Zeinab A. El-Sayed and Nesrine Radwan**

Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt

Primary immunodeficiency diseases (PIDs) are genetically inherited diseases characterized by an increased susceptibility to infections, autoimmunity, lymphoproliferation, and malignancies. PIDs are under-diagnosed and the registered cases and reported prevalence are far below the estimated numbers especially in countries with large population and high consanguinity rates. Delays in diagnosis yield major morbidities and mortalities with resultant increased economic burden. Newborn screening using TRECs and KRECs, currently being implemented in some countries, is aimed through early diagnosis, to overcome the delays in the diagnosis and hence the poor outcome of some of the severe PIDs. However, the limited resources in developing countries challenges the implementation of newborn PID screening programs. There are considerable gaps in our knowledge that must be bridged. Setting the norms of TRECs and KRECs for each country is needed. Furthermore, some PIDs that might present in the neonatal period could not be detected by the current screening programs, and their diagnosis requires clinical expertise. Not to mention, local guidelines for the management of patients diagnosed by NBS should be set forth. Also, in the absence of NBS, clinicians should be aware of the early manifestations of PID. All these mandate conducting studies genuine to each country, developing programs for raising public awareness and clinical training of physicians to attain the required immunological skills.

Keywords: newborn screening, primary immunodeficiency diseases, TRECs, KRECs, MENA region

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****Correspondence:***
Nesrine Radwan
nesrine_radwan@med.asu.edu.eg; nesrineradwan@yahoo.com

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INTRODUCTION

Primary immunodeficiency diseases (PIDs) are genetically inherited diseases that are characterized by an increased susceptibility to infections, autoimmunity, lymphoproliferation disorders, and malignancies (1). It was previously considered rare; however, recent studies showed that almost 1% of the population will have a PID (2, 3). The number of cases globally diagnosed in 2018 is 94,024 with an increase of 21.8% than in 2013 (3). The prevalence of PID varies from one region to another, being higher in the USA, followed by Europe, Latin America, Middle East, Asia, and finally Africa (3). Currently, more than 320 genes have been discovered to cause PID with a wide range of clinical phenotype (4).

PIDS: THE IMPACT OF UNDER AND DELAYED DIAGNOSIS

PIDs are usually detected in early childhood, but, in some cases, the diagnosis can be delayed (5). This can be attributed to diagnosis lag. The diagnosis lag, the time elapsing from the initial presentation to establishing a diagnosis, was reported to be prolonged, even with the most advanced healthcare systems, surprisingly reaching 12.4 years in the USA (6). In a recent worldwide survey on X-linked agammaglobulinemia (XLA), there was a wide variation in diagnosis lag, with 34% of the participating centers reporting delays beyond 2 years (7). This may be attributed to the lack of awareness, the scarcity of specialized centers, the poor resources for diagnosis, and, finally, the wide range of clinical phenotype.

The delay in diagnosis is of considerable concern with respect to the consequences of repeated and severe infections such as bronchiectasis and the complications resulting from live attenuated vaccinations such as BCG and oral poliomyelitis vaccine (OPV). Disseminated BCG-osis has been a well-known complication with a reported incidence of 64% among patients presenting with disseminated BCG-osis (8). OPV can replicate for a prolonged period in PID patients, which can cause an increase in transmissibility and neurovirulence of the virus, which, besides carrying the hazard of vaccine associated poliomyelitis in PID patients, can theoretically be a potential threat to the community (9). These negatively impact the morbidity and mortality of PIDs and the outcome of therapies including hematopoietic stem cell transplantation (HSCT).

NEWBORN SCREENING (NBS) FOR PIDS

There is a compelling need for having an early detection program, given that early detection of PID dramatically improves quality of life and life expectancy through prompt implementation of appropriate medical interventions and saves much of the expenses in medical care. Hence, neonatal screening was suggested for the purpose of early recognition of treatable, severe forms of PIDs with profoundly low T and B cell numbers (10, 11). This is done through quantifying T-cell receptor excision circles (TRECs) and kappa deleting-recombination excision circles (KRECs) (10).

TRECs are small circular DNA by-products produced during T cell receptor recombination in naïve T cells (12). They were found to be specific to naïve T cells and their levels decline with age in healthy individuals and HIV patients. TREC copy numbers are measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (13). They are reduced or absent in severe combined immunodeficiency (SCID) and other T cell lymphopenias (12). The assay is highly sensitive for detection of SCID with severe T cell lymphopenia, albeit not for SCID variants. The assay allows the identification of some other PIDs such as complete DiGeorge syndrome, leaky SCID, and ataxia telangiectasia (14). However, there are some PID reported to have normal TRECs (see **Table 1**). In 2005, the TREC assay was applied for large-scale screening in the USA (16), and nowadays, it is applied in most states. This led to a higher than expected incidence of SCID reaching 1/58,000 (17).

TABLE 1 | Causes of normal TRECs and KRECs.

Normal TRECS	Normal KRECS
Common variable immunodeficiency	IL2RA
Partial ADA SCID	JAK3
MHC Class II	CD40L
FOXP3	Selective IgA deficiency
CD40L	IL7RA
IL-10RA	

ADA, adenosine deaminase; CD40LG, CD40 ligand; CVID, common variable immunodeficiency; IgAD, IgA deficiency; IL10RA, interleukin 10 receptor, alpha; IL2RA, interleukin 2 receptor, alpha; IL2RG, interleukin 2 receptor, gamma; IL7RA, interleukin 7 receptor, alpha; JAK3, Janus kinase 3; KRECs, k-deleting recombination excision circles; SCID, severe combined immunodeficiency; TRECs, T-cell receptor excision circle. Quoted and modified from Serana et al. (15).

KRECs are B cell products produced during rearrangement of the variable, diversity, and joining domains of the B cell immunoglobulin kappa gene (18). It was first developed for assessment of patients with antibody deficiency disorders and monitoring B cell recovery following HSCT (19). In 2011, the utility of the KREC assay in identifying XLA and XLA-like diseases in neonates was demonstrated (18). However, there are instances of normal KRECs level happening in some PIDs (see **Table 1**).

In spite of the added costs by measuring both TRECs and KRECs level, it allows identification of more types of PIDs such as late onset ADA deficiency and some cases of Nijmegen breakage syndrome (20). Also, measuring them is considered less expensive than flow cytometry assay, which is not only expensive, but needs a lot of training and not available in all countries (21). Adding to the merits of NBS, the cost reduction calculated for early diagnosis was $85,882 and $55,882 for patients treated with immunoglobulin replacement therapy (3). Finally, the survival rate of infants transplanted before 3 months and diagnosed by NBS was 94% in comparison to those transplanted later and had infections (50%) (11, 22).

Caution is to be pursued while assessing the results of NBS. Preterm infants suffer from many problems, which yield the diagnosis using newborn screening challenging (10) and samples taken prior to the 32nd week of gestation to be repeated subsequently to exclude abnormal results due to physiological immaturity (23). Whether prematurity *per se* is a risk factor of having low TRECs or not is debatable (23). Multiple congenital anomalies and congenital heart disease are associated with low TREC levels (12). Maternal drug history is important since maternal intake of azathioprine was associated with low KREC level in offspring (24, 25). In addition, ethnic differences in cutoff levels of TRECs and KRECs exist and remain to be studied on a larger scale [(20, 23, 26–30); **Figure 1**].

The future is now heading toward protein-based screening methodologies to identify infants with complement and granulocyte disorders by measuring specific granulocytes proteins and some complement components using reverse-phase protein microarrays for determination of complement

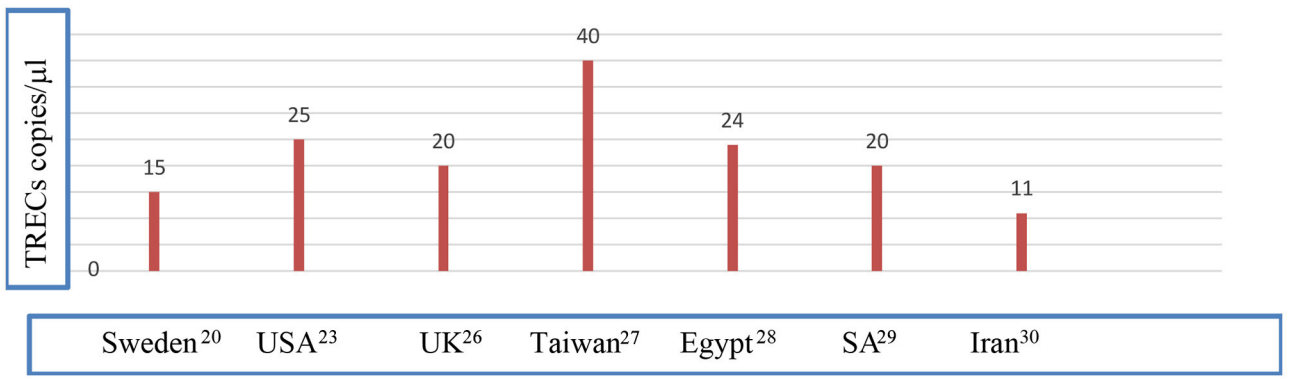

FIGURE 1 | Different cutoff values of TRECs in different populations. Data were quoted from Borte et al. (20), Kwan et al. (23), Adams et al. (26) Chien et al. (27), Salem et al. (28), Al-Mousa et al. (29), and Nourizadeh et al. (30). NBS, newborn screening; SA, Saudi Arabia; UK, United Kingdom; USA, United States of America.

component C3 levels in DBS collected at birth. Targeted DNA sequencing has previously been employed for screening selected diseases, such as glutaric acidemia type I and cystic fibrosis and has also been described as a potential screening method in familial hemophagocytic lymphohistiocytosis (FHLH) due to mutations in *UNC13D* (13).

MENA REGION AND THE CHALLENGES AHEAD

The incidence of consanguineous unions is almost 65% in the MENA region[1], which is significantly higher than in any other parts of the world (31). Consequently, the incidence of PID is expected to be 20 times greater in Middle Eastern countries compared with North America and Europe (32) as most PIDs are autosomal recessive diseases. However, the absence of national registries in most MENA region countries makes it difficult to determine the actual numbers. The estimated prevalence of PIDs in MENA region is 0.8–30.5/100,000, based on pilot studies and reports from different centers (32).

Based on the data collected from different publications (Egypt, Israel, Kuwait, Morocco, Saudi Arabia, Tunisia) (33–38), combined immunodeficiency (CID) ranks as the most common PID, whereas in Turkey (39), antibody deficiency is the most prevalent (32), and in the latest Iranian National registry too (40). The mean age of diagnosis is 2 years in the MENA region (32). A pilot study on NBS using TRECs and KRECs in Saudi Arabia showed an increase in the number of patients diagnosed to have SCID reaching an incidence of 1 in 2,906 live births (29), which is a much higher incidence than in California (1/65,000) live birth (41). This highlights the importance of implementing NBS for the early detection of SCID in MENA region countries. Currently, few countries of the MENA region have taken to newborn screening. These are Qatar in 2012 (12), Israel in 2015 (42), and Lebanon in 2018. UAE, Iran, and Turkey have taken some steps in this regard. (http://ipopi.org/).

[1]MENA countries include the following: Saudi Arabia, Kuwait, Bahrain, Qatar, United Arab Emirates, Oman, Yemen, Jordan, Iraq, Israel, Palestine, Syria, Lebanon, Iran, Turkey, Egypt, Sudan, Tunisia, Libya, Algeria, Morocco, and Mauritania.

PECULIARITIES OF AFRICA

Africa is a densely inhabited continent with an average inbreeding of around 35.4% (35) and surpassing 60% in parts of North and Sub-Saharan Africa (43). Although Africa is expected to have 988,000 PID cases, barely 2,500 patients have been diagnosed (36). The lack of resources, the absence of neonatal screening programs, and the need to enhance immunologic expertise are among the problems that hinder good medical care for PID. However, a steady rise in the number of reported PID cases is noticeable in between 2013 (1,463 patients) and 2018 (1,836 patients) with an increase rate of 25.6% (3). These advances are observed in countries where awareness programs are conducted such as Egypt, Tunisia, Algeria, Morocco, and Sudan (43).

STATUS IN EGYPT

Egypt is considered one of the largest countries in the MENA region and has the largest population. The university hospitals carry the main brunt of identification of PID cases. In 2010, with the implementation of the WHO program of surveillance for vaccine-derived poliovirus in PID patients (iVDPV) (44), the Ministry of Health and Population has become increasingly involved in the surveillance for PID cases. Data on the prevalence of PIDs in Egypt is lacking as there is no national registry to date. The ESID database contains data from Egypt and some information can also be gained from the few published studies (33, 45–47). CID is considered the most common disease (31%), followed by predominantly antibody deficiency (30%), well-defined syndromes with PID (17.5%), and, finally, phagocytic defects (8.1%). The diagnostic lag mean was 29.9 months (45) in 2008 and improved to 1.67 years in 2016 (33).

Egypt still uses oral polio vaccine (OPV). In 2018, a dose of IPV (inactivated poliovirus vaccine) was introduced at the age of 4 months to the routine immunization schedule of infants. All

newborns are given BCG and OPV at birth. This put, the as yet undiagnosed PID patient is at a significant risk of developing BCGosis and vaccine-associated paralytic poliomyelitis. In a study conducted on 130 patients with suspected or confirmed PID disorders, 6 patients were excreting VDPV, in which 5 of them had SCID and had X-linked agammaglobulinemia. Three patients developed acute flaccid paralysis (48). This underscores the importance of implementing the newborn screening programs, despite limited resources, for early diagnosis of PIDs and prevention of vaccination complications.

In a study on healthy Egyptian children, the level of TRECs and KRECs was found to be inversely proportional to age (46). The lowest threshold of TRECs (copies/μl blood) was 25 for the group aged 1 day to 5 months, and 24 for the group aged 5 months to 2.4 years. As for KRECs, the lowest threshold was 31 and 52, respectively (28).

DISCUSSION

PIDs are still underdiagnosed, and there is still a considerable diagnosis lag even in developed countries. Late diagnosis has major morbidity and mortality effects, and the establishment of screening programs for early detection and management becomes imperative.

In the absence of newborn screening programs for PIDs in many countries including Egypt, "clinical pattern recognition" (49) of various PIDs becomes a necessity, yet this would not supplant the need for neonatal screening programs. Many PIDs manifest in the neonatal period such as SCID, leucocyte adhesion deficiency (LAD), severe congenital neutropenia (SCN), chronic granulomatous disease (CGD), and defects in innate immunity. The diagnosis can be challenging at this age, and the difficulty, in part, stems from the natural immaturity of the neonatal immune system that may mask immune deficits and/or complicate interpretation of clinical findings and laboratory assays (50). The anatomic characteristics such as weak mucosal barriers, impaired Th1 cytokine production and immature cell-mediated immunity renders the newborn at risk of infection (50). The newborn has both qualitative and quantitative defects in complement (51). In sepsis, neutrophil count often falls due to exhaustion of the bone marrow reserves, which could be misdiagnosed as congenital neutropenia (51).

The most predictive factor for PID diagnosis is a family history of immunodeficiency, either confirmed or suspected, leading to early death or recurrent/chronic illness in one or more family members. Warning signs for PID in neonates have been suggested by the Jeffrey Modell Foundation (http://www.info4pi.org) and was modified by O'Connell (53). SCID patients could present with resistant or opportunistic infections. The presence of erythroderma should raise the suspicion of Omenn syndrome (54). The presence of eczema along with severe/recurrent infections in a neonate should raise the suspicion of certain diseases such as immunodeficiency polyendocrinopathy X-linked (IPEX), Wiskott-Aldrich syndrome (WAS), hyper-immunoglobulin E syndrome (HIES) (55), and DOCK 8 deficiency (56). Each of the previously described disease has an additional special character, such as microthrombocytopenia with WAS and staph infections with HIES. Mucosal abnormalities such as thrush and mouth ulcerations might mirror the underlying defect as STAT3 gain-of-function mutation. Delayed separation of the umbilical stump beyond 2 weeks is characteristic of LAD but may also be seen in other disorders such as IL-1 receptor kinase-4 (IRAK4) deficiency, SCN or CGD (51). Characteristic facial features along with cardiac defects with hypocalcemia suggest DiGeorge syndrome or CHARGE association (52). Thus, PID must be considered in the presence of certain syndromic features and abnormal facies. Presence of autoimmunity could be a clue for diseases such as IPEX. Enteritis in a neonate might be due to IL-10 and IL-10 receptor deficiencies (53).

A rare feature of PIDs is hydrops fetalis reported in IPEX (57) and X-linked lymphoproliferative disease type 2 (58). Hence, genetic testing is indicated in neonates with unexplained hydrops. Hepatosplenomegaly in the neonatal period has been described in hemophagocytic lymphohistiocytosis, and CGD (59). As confirmation of the importance of clinical identification of PID patients, the survival of patients diagnosed clinically was identical to those diagnosed by NBS, positive family history, or both (60).

Once a neonate is suspected of having a PID disorder, further investigations for the immune system should be done. Initial screening tests include complete blood counts. Quantitative Ig levels though are less informative in newborn and young infants because of reduced production and the transplacental transfer of IgGs. Lymphocyte subset enumeration by flow cytometry is indicated if SCID is suspected even in the setting of a normal lymphocyte count. The lymphoproliferative response to mitogens is the primary test for evaluating T cell function in newborns (61). In July 2019, the World Health Organization placed immunoglobulin quantitation and lymphocyte subset enumeration on the WHO list of essential *in vitro* diagnostics[2].

Finally, newborn screening is essential for early diagnosis and management of PIDs, reducing morbidities, and perhaps saving lives of some PIDs especially SCIDs. However, still there are no available screening tests for many other PIDs, and their diagnosis requires clinical expertise. Although the importance of implementing screening programs cannot be outweighed, the lack of resources dictates that educational programs and disease-focused awareness campaigns be adopted. Appropriate support should be given for the provision of immunological and molecular diagnostic tools. In the meantime, dedicated technical efforts and funds and harmonization of international cooperation to achieve alignment with national health agendas will help overcome problems with newborn screening programs.

AUTHOR CONTRIBUTIONS

ZE-S conceived this review. ZE-S and NR participated in searching and collecting the sources, drafting, and final approval of the manuscript.

[2]https://www.who.int/medical_devices/publications/Standalone_document_v8.pdf?ua=1 (accessed September 1, 2019).

REFERENCES

1. Raje N, Dinakar C. Overview of immunodeficiency disorders. *Immunol Allergy Clin North Am*. (2015) 35:599–623. doi: 10.1016/j.iac.2015.07.001
2. Boyle JM, Buckley RH. Population prevalence of diagnosed primary immunodeficiency diseases in the United States. *J Clin Immunol*. (2007) 27:497–502. doi: 10.1007/s10875-007-9103-1
3. Modell V, Orange JS, Quinn J, Modell F. Global report on primary immunodeficiencies: 2018 update from the Jeffrey Modell Centers Network on disease classification, regional trends, treatment modalities, and physician reported outcomes. *Immunol Res*. (2018) 66:367–80. doi: 10.1007/s12026-018-8996-5
4. Bousfiha A, Jeddane L, Picard C, Ailal F, Gaspar HB, Al-Herz W, et al. The 2017 IUIS *Phenotypic Classification for Primary Immunodeficiencies*. *J Clin Immunol*. (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8
5. Rossouw TM, Theron AJ, Anderson R. Practical approach to diagnosis and management of primary immunodeficiency diseases. *South Afr Fam Pract*. (2018) 60:42–9.
6. Orange JS, Seeborg FO, Boyle M, Scalchunes C, Hernandez-Trujillo V. Family physician perspectives on primary immunodeficiency diseases. *Front Med*. (2016) 3:12. doi: 10.3389/fmed.2016.00012
7. El-Sayed ZA, Abramova I, Aldave JC, Al-Herz W, Bezrodnik L, Boukari R, et al. X-linked agammaglobulinemia (XLA): phenotype, diagnosis, and therapeutic challenges around the world. *World Allergy Organ J*. (2019) 12:100018. doi: 10.1016/j.waojou.2019.100018
8. Bolursaz MR, Lotfian F, Velayati AA. *Bacillus Calmette-Guérin* vaccine complications in Iranian children at a University Hospital. *Allergol Immunopathol*. (2017) 45:356–61. doi: 10.1016/j.aller.2016.10.006
9. Kew OM, Sutter RW, de Gourville EM, Dowdle WR, Pallansch MA. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. *Annu Rev Microbiol*. (2005) 59:587–635 doi: 10.1146/annurev.micro.58.030603.123625
10. Jiang T, Li Z, Zhang Q. Advances in neonatal screening for primary immune deficiencies. *Exp Ther Med*. (2016) 11:1542–44. doi: 10.3892/etm.2016.3119
11. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000–2009. *N. Engl. J. Med*. (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
12. King J, Ludvigsson JF, Hammarström L. Newborn screening for primary immunodeficiency diseases: the past, the present and the future. *Int. J. Neonatal Screen*. (2017) 3:19. doi: 10.3390/ijns3030019
13. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. (1998) 396:690–5. doi: 10.1038/25374
14. Routes J, Abinun M, Al-Herz W, Bustamante J, Condino-Neto A, De La Morena MT, et al. ICON: The early diagnosis of congenital immunodeficiencies. *J Clin Immunol*. (2014) 34:398–424. doi: 10.1007/s10875-014-0003-x
15. Serana F, Chiarini M, Zanotti C, Sottini A, Bertoli D, Bosio A, Caimi L, Imberti L. 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) 911:119. doi: 10.1186/1479-5876-11-119
16. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol*. (2005) 115:391–8. doi: 10.1016/j.jaci.2004.10.012
17. King JR, Hammarström L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol*. (2018) 38:56–66. doi: 10.1007/s10875-017-0455-x
18. Nakagawa N, Imai K, Kanegane H, Sato H, Yamada M, Kondoh K, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. *J. Allergy Clin. Immunol*. (2011) 128:223–5. doi: 10.1016/j.jaci.2011.01.052
19. Van Zelm MC, Szczepanski T, Van der Burg M, Van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med*. (2007) 204:645–55. doi: 10.1084/jem.20060964
20. 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
21. Korsunskiy I, Blyuss O, Gordukova M, Davydova N, Gordleeva S, Molchanov R, Munblit D. TREC and KREC levels as a predictors of lymphocyte subpopulations measured by flow cytometry. *Front. in Physiolo*. (2019) 9:1877. doi: 10.3389/fphys.2018.01877
22. van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal Newborn Screening for Severe Combined Immunodeficiency (SCID). *Front Pediatr*. (2019) 18:373. doi: 10.3389/fped.2019.00373
23. 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) 312:729–38. doi: 10.1001/jama.2014.9132
24. 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) 37:51–60. doi: 10.1007/s10875-016-0347-5
25. 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) 27:70–7. doi: 10.1111/pai.12501
26. 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) 34:323–30. doi: 10.1007/s10875-014-0007-6
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) 114:12–6. doi: 10.1016/j.jfma.2012.10.020
28. Salem L, Talkhan HA, Bakr S, Mostafa AA, Mohamed NA. Simultaneous quantification of T-Cell Receptor Excision Circles (TRECs) and K-Deleting Recombination Excision Circles (KRECs) by real-time PCR. *Int J Adv Res*. (2016) 4:1251–8. doi: 10.21474/IJAR01/206
29. 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) 16:782. doi: 10.3389/fimmu.2018.00782
30. 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) 88:e12699. doi: 10.1111/sji.12699
31. Hadizadeh H, Salehi M, Khoramnejad S, Vosoughi K, Rezaei N. The association between parental consanguinity and primary immunodeficiency diseases: a systematic review and meta-analysis. *Pediatr Allergy Immunol*. (2017) 28:280–7. doi: 10.1111/pai.12685
32. Al-Mousa H, Al-Saud B. Primary immunodeficiency diseases in highly consanguineous populations from Middle East and North Africa: epidemiology, diagnosis, and care. *Front Immunol*. (2017) 8:678. doi: 10.3389/fimmu.2017.00678
33. Galal N, Meshaal S, Elhawary R, Abd ElAziz D, Alkady R, Lotfy S, et al. Patterns of primary immunodeficiency disorders among a highly consanguineous population: cairo university pediatric hospital's 5-year experience. *J Clin Immunol*. (2016) 36:649–55. doi: 10.1007/s10875-016-0314-1
34. Golan H, Dalal I, Garty BZ, Schlesinger M, Levy J, Handzel Z, et al. The incidence of primary immunodeficiency syndromes in Israel. *Isr Med Assoc J*. (2002) 4:868–71. doi: 10.1007/s10875-017-0378-6
35. Al-Herz W, Al-Ahmad M, Al-Khabaz A, Husain A, Sadek A, Othman Y. The Kuwait National Primary Immunodeficiency Registry 2004–2018. *Front Immunol*. (2019) 10:1754. doi: 10.3389/fimmu.2019.01754
36. Bousfiha AA, Jeddane L, El Hafidi N, Benajiba N, Rada N, El Bakkouri J, et al. First report on the Moroccan registry of primary immunodeficiencies: 15 years of experience. (1998–2012). *J Clin Immunol*. (2014) 34:459–68. doi: 10.1007/s10875-014-0005-8
37. Al-Saud B, Al-Mousa H, Al Gazlan S, Al-Ghonaium A, Arnaout R, Al-Seraihy A, et al. Primary immunodeficiency diseases in Saudi Arabia: a tertiary care hospital experience over a period of three years (2010–2013). *J Clin Immunol*. (2015) 35:651–60. doi: 10.1007/s10875-015-0197-6

38. Mellouli F, Mustapha IB, Khaled MB, Besbes H, Ouederni M, Mekki N, et al. Report of the Tunisian registry of primary immunodeficiencies: 25-years of experience (1988–2012). *J Clin Immunol*. (2015) 35:745–53. doi: 10.1007/s10875-015-0206-9
39. Kilic SS, Ozel M, Hafizoglu D, Karaca NE, Aksu, G., Kutukculer, N.The prevalence [correction] and patient characteristics of primary immunodeficiency diseases in Turkey – two centers study. *J Clin Immunol*. (2013) 33:74–83. doi: 10.1007/s10875-012-9763-3
40. Abolhassani H, Kiaee F, Tavakol M, Chavoshzadeh Z, Mahdaviani SA, Momen T, et al. Fourth update on the Iranian National Registry of Primary Immunodeficiencies: integration of molecular diagnosis. *J Clin Immunol*. (2018) 7:816–832. doi: 10.1007/s10875-018-0556-1
41. Puck JM. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. *Immunol Rev*. (2019) 287:241–252. doi: 10.1111/imr.12729
42. Rechavi E, Lev A, Saraf-Levy T, Etzioni A, Almashanu S, Somech R. Newborn screening for severe combined immunodeficiency in Israel. *Int. J. Neonatal Screen*. (2017) 3:13. doi: 10.3390/ijns3020013
43. El-Gamal YM, Hossny EM, El-Sayed ZA, Reda SM. Allergy and immunology in Africa: challenges and unmet needs. *J Allergy Clin Immunol*. (2017) 140:1240–3. doi: 10.1016/j.jaci.2017.09.004
44. El-Sayed ZA, Mach O, Hossny EM, Galal NM, El-Sawy I, Elmarsafy A, et al. Poliovirus excretion among persons with primary immune deficiency disorders: summary of data from enhanced poliovirus surveillance in Egypt, 2011–2014. *J Vaccines Vaccin*. (2016) 7:4. doi: 10.4172/2157-7560.1000331
45. Reda SM, Afifi HA, Amine MM. Amine AMM. primary immunodeficiency diseases in Egyptian children: a single-center study. *J Clin Immunol*. (2009) 29:343–351. doi: 10.1007/s10875-008-9260-x
46. Hussien SA MM, Mohammad MA, Zidan AA. Assessment of primary immunodeficiency disorders among children at Zagazig. *ZUMJ*. (2014) 20:296–306. doi: 10.21608/zumj.2014.4360
47. Galal N, Ohida M, Meshaal S, Elaziz DA, Elhawary I. Targeted screening for primary immunodeficiency disorders in the neonatal period and early infancy. *Afr Health Sci*. (2019) 19:1449–59. doi: 10.4314/ahs.v19i1.18
48. Galal NM, Meshaal S, ElHawary R, Nasr E, Bassiouni L, Ashghar H, et al. Poliovirus excretion following vaccination with live poliovirus vaccine in patients with primary immunodeficiency disorders: clinicians' perspectives in the endgame plan for polio eradication. *BMC Res Notes*. (2018) 11:717. doi: 10.1186/s13104-018-3822-7
49. De Vries E, Driessen G. Educational paper: primary immunodeficiencies in children: a diagnostic challenge. *Eur J Pediatr*. (2011) 170:169–77. doi: 10.1007/s00431-010-1358-5
50. Puck JM. Newborn screening for primary immunodeficiencies. Cited in Available online at: https://www.uptodate.com/contents/newborn-screening-for-primary-immunodeficiencies (accessed July 1, 2018).
51. Walkovich K, Connelly JA. Primary immunodeficiency in the neonate: early diagnosis and management. *Semin Fetal Neonatal Med*. (2016) 21:35–43. doi: 10.1016/j.siny.2015.12.005
52. Gupta V, Kumar A. Primary immunodeficiency disorders in Neonates, young infants and children: clinical diagnosis and management. *J Neon*. (2013) 27:31–5. doi: 10.1177/0973217920130306
53. O'Connell AE. Primary immunodeficiency in the NICU. *Neo Reviews*. (2019) 20:e68–77. doi: 10.1542/neo.20-2-e67
54. Gruber TA, Shah AJ, Hernandez M, Crooks GM, Abdel-Azim H, Gupta S, et al. Clinical and genetic heterogeneity in Omenn syndrome and severe combined immune deficiency. *Pediatr Transplant*. (2009) 13:244–50. doi: 10.1111/j.1399-3046.2008.00970.x
55. Woellner C, Gertz EM, Schaffer AA, Lagos M, Perro M, Glocker E-O, et al. Mutations in STAT3 and diagnostic guidelines for hyper-IgE syndrome. *J Allergy Clin Immunol*. (2010) 125:424–432 e428. doi: 10.1016/j.jaci.2009.10.059
56. Shah N, Kammermeier J, Elawad M, Glocker EO. Interleukin-10 and interleukin-10-receptor defects in inflammatory bowel disease. *Curr Allergy Asthma Rep*. (2012) 12:373–9. doi: 10.1007/s11882-012-0286-z
57. Reichert SL, McKay EM, Moldenhauer JS. Identification of a novel nonsense mutation in the FOXP3 gene in a fetus with hydrops: expanding the phenotype of IPEX syndrome. *Am J Med Genet A*. (2016) 170:226–32. doi: 10.1002/ajmg.a.37401
58. Lekbua A, Ouahed J, O'Connell AE, Kahn SA, Goldsmith JD, Imamura T, et al. Risk-factors associated with poor outcomes in VEO-IBD secondary to XIAP deficiency: a case report and literature review. *J Pediatr Gastroenterol Nutr*. (2019) 69:e13–8. doi: 10.1097/MPG.0000000000002297
59. Valentine G, Thomas TA, Nguyen T, Lai YC. Chronic granulomatous disease presenting as hemophagocytic lymphohistiocytosis: a case report. *Pediatrics*. (2014) 134:e1727–e30. doi: 10.1542/peds.2014-2175
60. Heimall J, Logan BR, Cowan MJ, Notarangelo LD, Griffith LM, Puck JM, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. *Blood*. (2017) 130:2718–27. doi: 10.1182/blood-2017-05-781849
61. Stiehm ER, Ochs HD, Winkelstein JA. Immunodeficiency disorders: general considerations. In: Stiehm ER, Ochs HD, Winkelstein JA, editors. *Immunologic disorders in Infants and Children,* 5th ed. Philadelphia, PA: Elsevier (2005). p. 311.

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Newborn Screening for Presymptomatic Diagnosis of Complement and Phagocyte Deficiencies

*Mahya Dezfouli[1,2], Sofia Bergström[2], Lillemor Skattum[3,4], Hassan Abolhassani[1,5], Maja Neiman[2], Monireh Torabi-Rahvar[6], Clara Franco Jarava[7], Andrea Martin-Nalda[8], Juana M. Ferrer Balaguer[9], Charlotte A. Slade[10,11], Anja Roos[12], Luis M. Fernandez Pereira[13], Margarita López-Trascasa[14], Luis I. Gonzalez-Granado[15], Luis M. Allende-Martinez[16], Yumi Mizuno[17], Yusuke Yoshida[18], Vanda Friman[19], Åsa Lundgren[20], Asghar Aghamohammadi[5], Nima Rezaei[5], Manuel Hernández-Gonzalez[7], Ulrika von Döbeln[21], Lennart Truedsson[3], Toshiro Hara[17], Shigeaki Nonoyama[18], Jochen M. Schwenk[2], Peter Nilsson[2] and Lennart Hammarström[1]**

[1] Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden, [2] Division of Affinity Proteomics, Department of Protein Science, KTH Royal Institute of Technology & SciLifeLab, Stockholm, Sweden, [3] Department of Laboratory Medicine, Section of Microbiology, Immunology and Glycobiology, Lund University, Lund, Sweden, [4] Clinical Immunology and Transfusion Medicine, Region Skåne, Lund, Sweden, [5] Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran, [6] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [7] Immunology Department, Vall d'Hebron Research Institute, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain, [8] Pediatric Infectious Diseases and Immunodeficiencies Unit, Vall d'Hebron Research Institute, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain, [9] Immunology, Hospital Universitari Son Espases/Institut d'Investigació Sanitària Illes Balears, Palma, Spain, [10] Royal Melbourne Hospital, Melbourne, VIC, Australia, [11] The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia, [12] Department of Microbiology and Immunology, Sint Antonius Hospital, Nieuwegein, Netherlands, [13] Department of Immunology, Hospital San Pedro de Alcántara, Cáceres, Spain, [14] Departamento de Medicina, Hospital La Paz Institute for Health Research (IdiPAZ), Universidad Autónoma de Madrid and Complement Research Group, Madrid, Spain, [15] Primary Immunodeficiencies Unit, Department of Pediatrics, University Hospital 12 de Octubre, Research Institute Hospital 12 Octubre (I+12), Madrid, Spain, [16] Immunology Department, University Hospital 12 de Octubre, Research Institute Hospital 12 Octubre (I+12), Madrid, Spain, [17] Fukuoka Children's Hospital, Kyushu University, Fukuoka, Japan, [18] Department of Pediatrics, National Defense Medical College, Saitama, Japan, [19] Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [20] Departments of Infectious Diseases, Central Hospital, Kristianstad, Sweden, [21] Division of Metabolic Diseases, Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm, Sweden

The clinical outcomes of primary immunodeficiencies (PIDs) are greatly improved by accurate diagnosis early in life. However, it is not common to consider PIDs before the manifestation of severe clinical symptoms. Including PIDs in the nation-wide newborn screening programs will potentially improve survival and provide better disease management and preventive care in PID patients. This calls for the detection of disease biomarkers in blood and the use of dried blood spot samples, which is a part of routine newborn screening programs worldwide. Here, we developed a newborn screening method based on multiplex protein profiling for parallel diagnosis of 22 innate immunodeficiencies affecting the complement system and respiratory burst function in phagocytosis. The proposed method uses a small fraction of eluted blood from dried

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National Institute of Pediatrics, Mexico

***Correspondence:**
Lennart Hammarström
lennart.hammarstrom@ki.se

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blood spots and is applicable for population-scale performance. The diagnosis method is validated through a retrospective screening of immunodeficient patient samples. This diagnostic approach can pave the way for an earlier, more comprehensive and accurate diagnosis of complement and phagocytic disorders, which ultimately lead to a healthy and active life for the PID patients.

Keywords: primary immunodeficiency, complement deficiencies, phagocytic disorders, presymptomatic diagnosis, newborn screening, dried blood spot, protein profiling

INTRODUCTION

Primary immunodeficiencies (PIDs) are a group of inherited disorders caused by defects in different components of the immune system. PIDs may be associated with severe clinical outcomes if left undiagnosed or untreated (1). However, it is uncommon to consider PIDs before the manifestation of severe clinical symptoms. Recently, novel newborn screening assays have been explored to enable early clinical intervention and disease management, beginning as early as the presymptomatic stage at birth. Newborn screening programs based on the use of dried blood spot (DBS) samples have revolutionized public healthcare by detecting disorders such as phenylketonuria (PKU) during the first few days of life (2). Further developments of this strategy introduced mass spectrometry (MS)-based screening with improved sensitivity, specificity and capacity (2). Most recently, DNA-based screening methodologies have been developed to detect severe PIDs with defects in adaptive immunity (2). PCR-based screening is being applied for diagnosing a subset of life-threatening PIDs, including severe combined immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA), through the measurement of episomal excision products of lymphocyte receptors in DBS samples (3). Selected countries have successfully implemented SCID-screening and several additional countries worldwide are considering to include it in their national screening programs (2).

Here, we present a newborn screening based on protein profiling that broadens the diagnosis to cover other severe forms of PIDs with innate immunity defects. The screening enables parallel diagnosis of 22 disorders due to defects in the complement system or phagocytic function prior to the onset of clinical symptoms. Complement deficiencies represent 1–29% of all PID cases, (4) giving a cumulative prevalence of 1 in 20,000 live births (5), and phagocytic disorders encompass 5–29% of PIDs, occurring at a prevalence of 1 in 250,000 live births (1, 6). However, due to the lack of comprehensive and pre-symptomatic diagnosis, the prevalence rate of these PIDs is markedly underestimated (7). Accurate and early diagnosis is vital as complement deficiencies and phagocytic disorders are associated with numerous immunological complications. Complement deficiencies cause recurrent and persistent infections in the upper respiratory tract, recurrent pneumococcal and meningococcal infections, hereditary angioedema (HAE), autoimmune complications, and renal failure due to atypical hemolytic uremic syndrome (aHUS) and glomerulonephritis (GN) (8). Severe congenital neutropenia (SCN) and chronic granulomatous disease (CGD) are characterized by low granulocyte counts or defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway, leading to conditions such as delayed wound healing, deep organ infections, and abscess formation. These infections may become severe or even lethal, and might lead to development of malignancies or bone marrow failure (6). Early diagnosis of such disorders allows immediate clinical intervention and prevention of severe complications. Moreover, in the case of phagocytic diseases, it might allow for early stem cell transplantation, with a future prospect of gene therapy.

Currently, complement functional assays, nephelometry, immunoprecipitation (IP), enzyme-linked immunosorbent assay (ELISA), complete blood count and flow-cytometric cellular characterizations are applied for diagnosis of complement and phagocytic disorders (5). All these methods are low-throughput, hardly adaptable to population-scale performance, and can be costly and labor-intensive to test for several disorders in one sample. Moreover, the small quantity of blood material in DBS significantly limits the total number of possible tests on each sample (9). Accordingly, an alternative diagnostic method that enables multiple parallel tests and requires minimal sample volume would greatly improve the clinical procedures, particularly in newborn screening. Considering the disease mechanisms of complement and phagocytic defects that involve the absence, or expression/structural alteration of a functional protein, protein profiling of blood samples could provide a fundamental diagnostic tool (10). Here, we developed a newborn screening assay based on suspension bead array technology for parallel profiling of the main reported disease-associated proteins in the complement cascade, phagocytosis and respiratory burst function (8). The method uses a fraction of DBS corresponding to a sub-microliter volume of blood, and is applicable for population-scale performance (**Figure 1**).

METHODS

Blood Samples and Preparation for Protein Profiling

Thirty-seven anonymized healthy control samples in forms of dried blood spot (DBS), serum, and whole blood (including 21 newborn samples) were provided by the Karolinska University Hospital Huddinge (Sweden) for normal range identification of the targeted proteins (see Antibodies and Immunoassay Procedure sections). Moreover, 41 DBS and

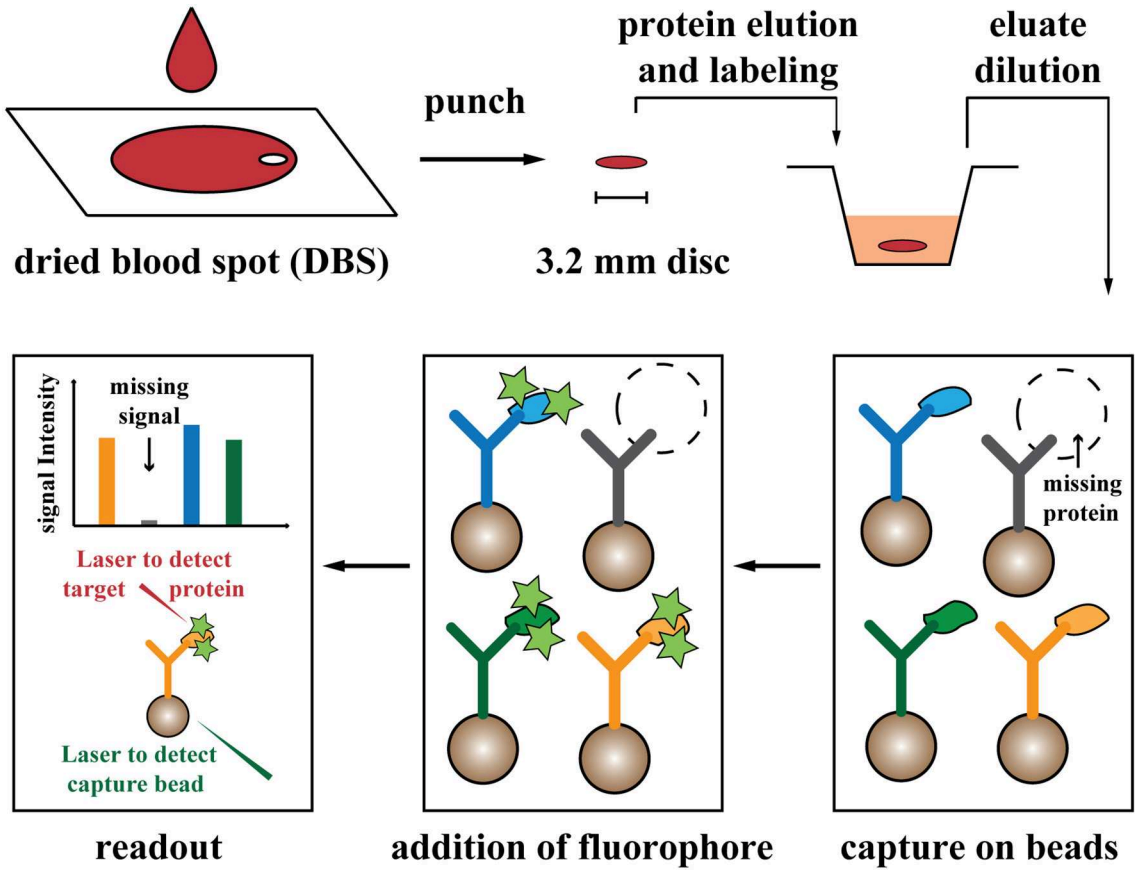

FIGURE 1 | Schematic view of the screening procedure. Drops of blood (~70 μL) are dried on filter paper. Proteins are eluted and labeled from a punched disc, equal to a 4.5% fraction of one blood drop. After further dilution (1:500 dilution of blood), proteins are captured on the designed antibody-coupled bead array, and signals are analyzed with fluorescent-based readout. Stars illustrated fluorescent label. Two laser beams are applied for detection of beads (capture antibodies) and associated fluorescent signal (from labeled target proteins).

matched serum samples (including five original Guthrie cards from the Newborn Screening Laboratory, Karolinska University Hospital, Sweden) with defects in one of the proteins C1, C2, C3, C4, C5, C6, C7, C8, C9, FB, FD, FH, FI, properdin, or HAX1 were obtained and prepared for retrospective screening (**Table 1**). All human samples used in this study were collected after informed consent and handled under the approval of the Regional Ethical Review Board (EPN) in Stockholm, Sweden.

Filter papers (PerkinElmer) for DBS sampling were provided by the Newborn Screening Laboratory at the Karolinska University Hospital Solna (Sweden), and were prepared by applying 70 μL (corresponding to one drop) fresh and intact whole blood onto the filter paper. Papers were dried at ambient temperature (25°C) for 4 h, before desiccated storage at 4°C until use. DBS samples were punched in 3.2 mm in diameter discs (corresponding to 4.5% fraction of one blood drop), and proteins were eluted by submerging the disc in 30 μL sterile phosphate buffered saline supplemented with Tween 20 (PBS-T, pH 7.4, 0.1% Tween 20, Medicago-AB) for 3 days, 1 day, or 2 h at 4°C. Subsequently, proteins from the DBS eluate or 3 μL matched whole blood were biotinylated (referred as protein labeling) by addition of ten times molar excess of Sulfo-N-Hydroxysuccinimide-polyethyleneglycol biotin (NHS-PEG4-Biotin, ThermoFisher Scientific, USA) in 30 μL PBS-T (referred as labeling solution), with incubation for 2 h at 4°C. Alternatively, a rapid and simultaneous protein elution and labeling was performed by submerging the DBS disc in 30 μL of labeling solution for 2 h, 30 min, or 10 min at ambient temperature. Labeled samples were stored at −20°C until use. Immediately before incubation with the suspension bead array, 1 μL of labeled samples were further diluted 50 times in PBS buffer supplemented with polyvinylpyrrolidone, polyvinyl alcohol, and casein (Sigma-Aldrich). The diluted samples were heat-treated at 56°C for 30 min for signal enhancement (10) and were cooled down to ambient temperature for 10 min prior use. The effect of heat-treatment on heat-labile complement proteins with no effect on protein profiling were previously described (10). The final 1:500 dilution of eluted samples from each disc provides enough volume for 30 tests, which corresponds to a 0.1 μL volume of crude whole blood applied to each multiplexed measurement.

TABLE 1 | Information on the sample cohort used in retrospective screening.

Diagnosed PID/disorder	Number of patient samples	Previous citation (if available)	Provided by
C1 deficiency	1#		Sigma-Aldrich, product# 234401
C2 deficiency	8 (five newborns)*	(10, 11)	Vall d'Hebron University Hospital, Spain Son Espases University Hospital, Spain 12 Octubre Hospital, Spain Karolinska University Hospital, Sweden
C3 deficiency	2#	(10)	Sigma-Aldrich, product# C8788 Karolinska University Hospital, Sweden
C4 deficiency	1#	(10)	Karolinska University Hospital, Sweden
C5 deficiency	1	(12)	Vall d'Hebron University Hospital, Spain
C6 deficiency	2		Sigma-Aldrich, product# C1288 La Paz University Hospital, Spain
C7 deficiency	3	(13)	Son Espases University Hospital, Spain Sahlgrenska University Hospital, Sweden National Defense Medical College, Japan
C8 deficiency	1#		Sigma-Aldrich, product#C1538
C9 deficiency	4	(14)	Fukuoka Children's Hospital, Japan National Defense Medical College, Japan
FB deficiency	1	(15)	Royal Melbourne Hospital, Australia
FD deficiency	3	(16)	Sint Antonius Hospital, the Netherlands
FH deficiency	2#	(10)	Karolinska University Hospital, Sweden
FI deficiency	8	(17, 18)	Vall d'Hebron University Hospital, Spain Royal Melbourne Hospital, Australia San Pedro de Alcántara Hospital, Spain 12 Octubre Hospital, Spain
Properdin deficiency	2		Royal Melbourne Hospital, Australia Central Hospital, Kristianstad, Sweden
SCN (HAX1 deficiency)	2	(19)	Tehran University of Medical Sciences, Iran
Total number of patients	41		See above
Healthy Controls	37 (21 newborns)		Karolinska University Hospital, Sweden

**Original Guthrie cards from newborn screening program in Sweden.*
#Available sample type is serum.

Antibodies and Immunoassay Procedure

Antibodies targeting the proteins and protein fragments C1qA, C1qB, C1s, C2b, C3, C3a, C4A, C4B, C5, C6, C7, C8A, C8B, C9, FB, FD, FH, FI, Properdin, CSF3R, p22-phox, gp91-phox, p47-phox, p67-phox, p40-phox, ELANE, HAX1, human albumin and human IgG were covalently coupled to carboxylated fluorescent-color-coded Luminex MagPlex microspheres (Luminex Corporation) according to a previously established protocol (20). In brief, 500,000 microspheres (referred to as beads) were functionalized using N-Hydroxysuccinimide-polyethyleneglycol (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemical linkers (ThermoFisher-Scientific) according to the manufacturer's protocol, and incubated with 1.65 μg of the antibody or recombinant protein in 100 μL 2-(N-Morpholino) ethanesulfonic acid (MES) buffer at pH 5.0 (Sigma-Aldrich) for 2 h at ambient temperature on a microplate shaker (650 rpm). Remaining active groups on the beads were quenched by an overnight incubation in 50 μL blocking reagent for ELISA (Roche). Antibody-coupled beads were pooled in suspension to be used as the suspension bead array in subsequent experiments. An array of 42 antibodies targeting the 22 proteins and their corresponding protein fragments was used for protein profiling (**Table S1**).

Fifty microliter of labeled, diluted, and heat-treated samples were applied to the prepared suspension bead array with bead counts of minimum 30 beads per target per measurement. After overnight incubation in a sealed plate at ambient temperature on a microplate shaker (650 rpm), the beads were washed three times in 100 μL PBS-T. The sandwich immunoassay was performed as described above, with the exception of using unlabeled DBS eluate, and addition of an incubation step with biotinylated secondary antibodies for 1 h at ambient temperature, followed by three times washes in 100 μL PBS-T (21). Subsequently, the captured biotin-labeled proteins on beads (or detection antibodies in sandwich format) were incubated with 50 μL of Streptavidin R-phycoerythrin conjugate (SAPE, ThermoFisher-Scientific) at the concentration of 5 μg/mL. After three times washes in 100 μL PBS-T and resuspension in 100 μL PBS-T, fluorescent signal intensities were measured in parallel for all targets using Luminex LX200 and

FLEXMAP 3D systems (Luminex Corporation) according to the manufacturer's instructions.

Data Analysis

R software version 3.2.3 and R Studio environment version 0.99.902 were applied for statistical analyses and data visualization (22). The median fluorescence intensities (MFI) from the beads regarding each protein target per measurement were used. The signal-to-background ratio was determined according to the internal negative control (data from beads with no capture antibody). Data were normalized and fluorescent signals were adjusted using the "batch effect removal" function from the R Limma package (23). The statistical cut-off for the definition of protein defects (PID level) was considered as an outlier fluorescent intensity under minimum values that deviates from the 1.5 times interquartile range (IQR) under the first quartile of corresponding signals for each protein measurement in all healthy control samples. Values from PID patients associated with non-targeted proteins were not considered as healthy values, as no previous clinical data is available for non-targeted proteins and deficiency in one complement protein can cause perturbation in the levels of other proteins due to regulatory, compensation and feedback mechanisms. The coefficient of variation (CV) was calculated for the assay as the ratio of the standard deviation to the mean for triplicate measurements from three different samples, two different sample material and 43 antibodies.

RESULTS

Initially, we confirmed protein elution from filter paper of three healthy controls, evaluated the protein measurements, as well as testing automation and reduction in turnaround time (**Figure S1A–C**). The procedure was set to 30 min protein elution and labeling at ambient temperature, followed by an overnight protein capture on an antibody-coupled bead array, and parallel fluorescent-based readout for 30 s per sample on the following day. The median coefficient of variation (CV) of the method was calculated to be 15. An optimal limit for blood dilution of above 1:800 was preferred to allow the detection of as many proteins as possible.

Subsequently, screening was performed on a sample cohort, including 37 healthy controls and 41 cases diagnosed with PID (**Table 1**). The cohort included 26 newborn samples, including five PID cases of original Guthrie cards from the Newborn Screening Laboratory at Karolinska Hospital (Sweden) and 21 healthy DBS samples provided by the National Cord Blood Biobank at Karolinska University Hospital Huddinge (Sweden). Additionally, these five original Guthrie card samples from newborns with PID were compared with healthy DBS samples for confirmation of the newborn screening concept.

Samples were profiled for complement components C1-C9, complement factors FB, FD, FH, FI, and properdin, as well as the proteins related to phagocytosis and granulocyte function, including colony-stimulating factor 3 receptor (CSF3R), phagocytic oxidase subunits p22-phox, gp91-phox, p47-phox, p67-phox, and p40-phox, neutrophil elastase (ELANE) and HCLS1 associated protein X-1 (HAX1) (**Table S1**). The deficiency level for each protein was defined based on the population of 37 healthy samples, as those with a deviation from healthy control values within the interquartile range of 1.5 (IQR $\geq$ 1.5). Data obtained from the retrospective screening confirmed the documented disorders within the analyzed cohort (**Figure 2**, **Figure S2**), mainly in DBS samples collected from neonates with PID, compared to normal newborn samples or healthy adults (**Table 1**). Furthermore, the signals obtained from deficient samples were shown to be regained by supplementing the corresponding recombinant protein into the DBS samples collected from the respective deficient donors (**Figure S1D**). The second-tier screening was also shown to be accessible in the form of sandwich immunoassays, allowing for a more selective detection of the target proteins (**Figure S3**). Lastly, the screening data were further validated by current standard clinical tests in matched serum samples of the PID cohort (**Table S2**).

DISCUSSION

Newborn screening programs are required to meet a panel of defined criteria for the principles and practice of each diagnostic method to be considered at a public health measure. The main criteria are that the disorder needs to be a serious health issue and late-stage treatment would cause increased morbidity and mortality, while early diagnosis would lead to a significantly better outcome, a treatment or effective preventive care should be readily available for the diagnosed condition, the disorder should have a high enough incidence among the target population, the costs involved should be economically balanced to adapt for massive population screening, and the turnaround time from sampling to data should not exceed 48 h (2).

The genetic disorders of the innate immunity are very serious, particularly when considering life-threatening infections and angioedema. Late diagnosis would cause significantly higher morbidity leading to permanent damage to vital organs and even death. A strict preventive care such as prophylactic antibody therapy, preventive treatment for systemic lupus erythematosus (SLE) and HAE, as well as postponing BCG vaccination and early hematopoietic stem cell transplantation (HSCT) in CGD patients would significantly reduce morbidity and mortality. Currently, the cumulative prevalence of deficiencies of the innate immunity, which could be markedly underestimated due to lack of accurate diagnosis, is within the range for newborn screening (1 in <20,000 live births in the target population). The cost per assay for the proposed method is below 5 USD for the detection of the 22 disorders (including the costs for all reagents and laboratory consumables) and is shown to be in the range of newborn screening guidelines for massive population screening. The turnaround time from sampling to data is fewer than 10 h and fits well with the time standard.

A key benefit of the developed screening system is the notable number of PID-associated proteins that can be examined in parallel using a limited amount of sample material. Moreover, the flexible design of the bead array content enables an optimal adaptation to national programs of different countries, since

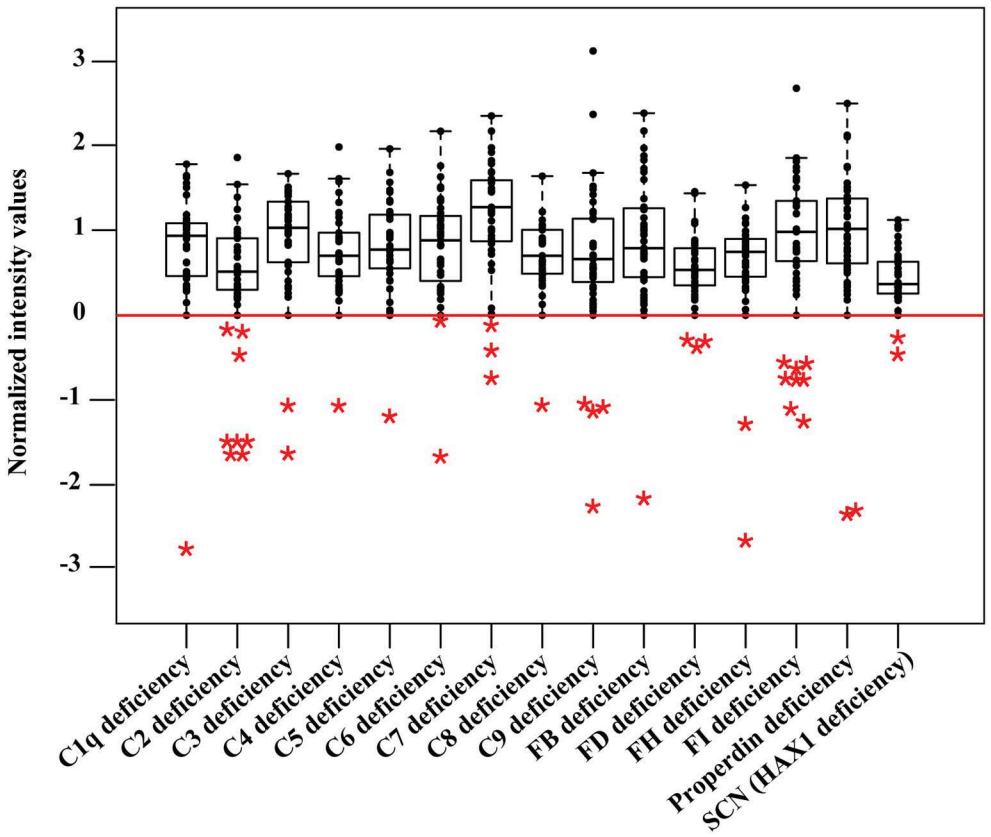

FIGURE 2 | Data from retrospective PID screening. Protein profiling is done in parallel in a multiplexed measurement and obtained values are shown in separate boxplots for different disorders from 37 healthy control samples including 21 newborn dried blood spots. The deficiency level (IQR $\geq$ 1.5) is shown as a red baseline. Deficient samples ($n = 41$) are shown in colored asterisks, including five newborn dried blood spots of original Guthrie cards from newborn screening program in Sweden. SCN stands for severe congenital neutropenia.

the prevalence and mechanism of PIDs are markedly different among ethnic groups. Nevertheless, the presented screening might miss identifying cases of rare, newly discovered types of PIDs, or disorders caused by a dysfunctional protein expression. Updating the array to include particular antibodies that bind the mutated, truncated, or distorted protein fragments might further identify these additional PID cases. The bead array technology can increase the sample throughput up to 384 individuals per instrument run, with a readout capacity of up to over 500 proteins in parallel (10, 20). We have shown the possibility for an extended array design to reach beyond the presented 22 disorders, by profiling over 1,000 protein targets in DBS samples (**Figure S4**).

Taken together, combining the long-established routines for DBS neonatal sampling with the inherent potential of multiplexed technologies offers an appealing avenue that can be readily expanded to nationwide newborn screening for a broader range of PIDs. In the future perspective, a pilot program would address the aspects of population-scale screening prior to the decision of implementation in the national screening programs. In addition, the actual prevalence rates of these PIDs will be adjusted based on data from the pilot program. As an instance, the prevalence of severe combined immunodeficiency (SCID) in the US was significantly adjusted from 1 in 100,000 to 1 in 58,000 live births before and after the pilot newborn screening study (24). Although newborn screening of severe diseases like CGD is urgently required, the pilot screening will confirm the usefulness of the screening for each country and will support the governmental decisions on the set of other innate PIDs to include in the nation-wide screening program. Since our current suggested method is flexible, depending on the prevalence of PIDs in a specific country the cost-effectiveness assessments in their health-care systems can be performed to designed required custom array. It is estimated that the innate immunodeficiency screening would benefit 20 newborns per year in Sweden and 6,500 newborns worldwide.

The current study should be considered a "proof of principle" and the precursor for a pilot study with enough samples size to make a decision as to cost/benefit and feasibility. Importantly, the presented approach could pave the way for an earlier, more comprehensive and accurate diagnosis of complement and phagocytic disorders. Correct and precise diagnosis with an insight over several conditions could inform a better understanding of these diseases by finding patients with specific or combination of deficiencies, in addition to showing

the differences between non-functional vs. abrogated protein expressions. With a more personalized diagnosis at hand, insights from multi-parameter assays can contribute to a more effective treatment and preventive care, which ultimately leads to a healthy and active life for PID patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethical Review Board (EPN) in Stockholm, Sweden. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MD, SB, LS, HA, MN, JS, PN, and LH conceived and designed the study. MD and SB planned and performed the laboratory experiments and acquisition of data in screening. LS designed and performed the validation. MD, SB, LS, HA, MT-R, JS, PN, and LH analyzed and interpreted the data and revised the manuscript critically. LS, HA, CF, JF, CS, AR, LF, ML-T, LG-G, LA-M, YM, YY, VF, ÅL, AA, NR, AM-N, MH-G, UD, LT, TH, SN, JS, and PN contributed with patient diagnosis and/or providing of reagents, materials, analysis tools. MD wrote the paper. All authors approved the final manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00455/full#supplementary-material

REFERENCES

1. Skattum L, van Deuren M, van der Poll T, Truedsson L. Complement deficiency states and associated infections. *Mol Immunol*. (2011) 48:1643–55. doi: 10.1016/j.molimm.2011.05.001
2. King JR, Hammarstrom L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol*. (2018) 38:56–66. doi: 10.1007/s10875-017-0455-x
3. 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
4. Rezaei N, Notarangelo LD, Aghamohammadi A. *Primary Immunodeficiency Diseases*. Berlin: Springer (2008).
5. Sjoholm AG, Jonsson G, Braconier JH, Sturfelt G, Truedsson L. Complement deficiency and disease: an update. *Mol Immunol*. (2006) 43:78–85. doi: 10.1016/j.molimm.2005.06.025
6. Lim MS, Elenitoba-Johnson KS. The molecular pathology of primary immunodeficiencies. *J Mol Diagn*. (2004) 6:59–83. doi: 10.1016/S1525-1578(10)60493-X
7. Grumach AS, Kirschfink M. Are complement deficiencies really rare? Overview on prevalence, clinical importance and modern diagnostic approach. *Mol Immunol*. (2014) 61:110–7. doi: 10.1016/j.molimm.2014.06.030
8. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, 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). doi: 10.1007/s10875-019-00737-x. [Epub ahead of print].
9. Ignjatovic V, Pitt J, Monagle P, Craig JM. The utility of dried blood spots for proteomic studies: looking forward to looking back. *Proteom Clin Appl*. (2014) 8:896–900. doi: 10.1002/prca.201400042
10. Hamsten C, Skattum L, Truedsson L, von Dobeln U, Uhlen M, Schwenk JM, et al. Heat differentiated complement factor profiling. *J Proteom*. (2015) 126:155–62. doi: 10.1016/j.jprot.2015.05.027
11. Turley AJ, Gathmann B, Bangs C, Bradbury M, Seneviratne S, Gonzalez-Granado LI, et al. Spectrum. and. management. of. complement. immunodeficiencies. (excluding hereditary angioedema) across Europe. *J Clin Immunol*. (2015) 35:199–205. doi: 10.1007/s10875-015-0137-5
12. Colobran R, Franco-Jarava C, Martin-Nalda A, Baena N, Gabau E, Padilla N, et al. Novel mutations causing C5 deficiency in three North-African Families. *J Clin Immunol*. (2016) 36:388–96. doi: 10.1007/s10875-016-0275-4
13. Horiuchi T, Ferrer JM, Serra P, Matamoros N, Lopez-Trascasa M, Hashimura C, et al. A novel nonsense mutation at Glu-631 in a Spanish family with complement component 7 deficiency. *J Hum Genet*. (1999) 44:215–8. doi: 10.1007/s100380050146
14. Nagata M, Hara T, Aoki T, Mizuno Y, Akeda H, Inaba S, et al. Inherited deficiency of ninth component of complement: an increased risk of meningococcal meningitis. *J Pediatr*. (1989) 114:260–4. doi: 10.1016/S0022-3476(89)80793-0
15. Slade C, Bosco J, Unglik G, Bleasel K, Nagel M, Winship I. Deficiency in complement factor B. *N Engl J Med*. (2013) 369:1667–9. doi: 10.1056/NEJMc1306326
16. Biesma DH, Hannema AJ, van Velzen-Blad H, Mulder L, van Zwieten R, Kluijt I, et al. A family with complement factor D deficiency. *J Clin Invest*. (2001) 108:233–40. doi: 10.1172/JCI200112023
17. Alba-Dominguez M, Lopez-Lera A, Garrido S, Nozal P, Gonzalez-Granado I, Melero J, et al. Complement factor I deficiency: a not so rare immune defect: characterization of new mutations and the first large gene deletion. *Orphanet J Rare Dis*. (2012) 7:42. doi: 10.1186/1750-1172-7-42
18. Franco-Jarava C, Colobran R, Mestre-Torres J, Vargas V, Pujol-Borrell R, Hernandez-Gonzalez M. Clinical laboratory standard capillary protein electrophoresis alerted of a low C3 state and lead to the identification of a

Factor I deficiency due to a novel homozygous mutation. *Immunol Lett*. (2016) 174:19–22. doi: 10.1016/j.imlet.2016.04.011
19. Klein C, Grudzien M, Appaswamy G, Germeshausen M, Sandrock I, Schaffer AA, et al. HAX1. deficiency. causes. autosomal. recessive. severe. congenital. neutropenia. (Kostmann disease). *Nat Genet*. (2007) 39:86–92. doi: 10.1038/ng1940
20. Schwenk JM, Gry M, Rimini R, Uhlén M, Nilsson P. Antibody suspension bead arrays within serum proteomics. *J Proteome Res*. (2008) 7:3168–79. doi: 10.1021/pr700890b
21. Dezfouli M, Vickovic S, Iglesias MJ, Nilsson P, Schwenk JM, Ahmadian A. Magnetic bead assisted labeling of antibodies at nanogram scale. *Proteomics*. (2014) 14:14–8. doi: 10.1002/pmic.201300283
22. R Core Team. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*, Vienna (2013). Available online at: http://www.R-project.org/
23. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. (2015) 43:e47. doi: 10.1093/nar/gkv007
24. 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) 312:729–38. doi: 10.1001/jama.2014.9133

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ORIGINAL RESEARCH
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EuroFlow Standardized Approach to Diagnostic Immunopheneotyping of Severe PID in Newborns and Young Children

Tomas Kalina[1†], Marina Bakardjieva[1†], Maartje Blom[2], Martin Perez-Andres[3], Barbara Barendregt[4], Veronika Kanderová[1], Carolien Bonroy[5,6], Jan Philippé[5,6], Elena Blanco[3], Ingrid Pico-Knijnenburg[2,4], Jitse H. M. P. Paping[2], Beata Wolska-Kuśnierz[7], Malgorzata Pac[7], Jakub Tkazcyk[8], Filomeen Haerynck[9], Himmet Haluk Akar[10], Renata Formánková[1], Tomáš Freiberger[11,12], Michael Svatoň[1], Anna Šedivá[13], Sonia Arriba-Méndez[14], Alberto Orfao[3], Jacques J. M. van Dongen[15] and Mirjam van der Burg[2,4]*

[1] Department of Paediatric Haematology and Oncology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czechia, [2] Laboratory for Immunology, Department of Pediatrics, Leiden University Medical Center (LUMC), Leiden, Netherlands, [3] Department of Medicine-Serv. Cytometry, Cancer Research Center (IBMCC-CSIC/USAL), University of Salamanca, Salamanca, Spain, [4] Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, Netherlands, [5] Department of Diagnostic Sciences, Ghent University, Ghent, Belgium, [6] Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium, [7] Department of Immunology, Children's Memorial Health Institute, Warsaw, Poland, [8] Department of Pediatrics, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czechia, [9] PID Research Lab, Department of Pediatric Pulmonology and Immunology, Ghent University Hospital, Ghent, Belgium, [10] Department of Pediatric Immunology and Allergy, Kanuni Sultan Süleyman Training and Research Hospital, Istanbul Health Sciences University, Istanbul, Turkey, [11] Centre for Cardiovascular Surgery and Transplantation, Brno, Czechia, [12] Medical Faculty, Masaryk University, Brno, Czechia, [13] Department of Immunology, University Hospital Motol, Prague, Czechia, [14] Servicio de Pediatría, Hospital Universitario de Salamanca, Salamanca, Spain, [15] Department of Immunohematology and Blood Transfusion (IHB), Leiden University Medical Center (LUMC), Leiden, Netherlands*

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***Correspondence:**
Mirjam van der Burg
m.van_der_burg@lumc.nl

[†]These authors have contributed equally to this work

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The EuroFlow PID consortium developed a set of flow cytometry tests for evaluation of patients with suspicion of primary immunodeficiency (PID). In this technical report we evaluate the performance of the SCID-RTE tube that explores the presence of recent thymic emigrants (RTE) together with T-cell activation status and maturation stages and discuss its applicability in the context of the broader EuroFlow PID flow cytometry testing algorithm for diagnostic orientation of PID of the lymphoid system. We have analyzed peripheral blood cells of 26 patients diagnosed between birth and 2 years of age with a genetically defined primary immunodeficiency disorder: 15 severe combined immunodeficiency (SCID) patients had disease-causing mutations in *RAG1 or RAG2* ($n = 4$, two of them presented with Omenn syndrome), *IL2RG* ($n = 4$, one of them with confirmed maternal engraftment), *NHEJ1* ($n = 1$), *CD3E* ($n = 1$), *ADA* ($n = 1$), *JAK3* ($n = 3$, two of them with maternal engraftment) and *DCLRE1C* ($n = 1$) and 11 other PID patients had diverse molecular defects [*ZAP70* ($n = 1$), *WAS* ($n = 2$), *PNP* ($n = 1$), *FOXP3* ($n = 1$), del22q11.2 (DiGeorge $n = 4$), *CDC42* ($n = 1$) and *FAS* ($n = 1$)]. In addition, 44 healthy controls in the same age group were analyzed using the SCID-RTE tube in four EuroFlow laboratories using a standardized 8-color approach. RTE were defined as CD62L+CD45RO-HLA-DR-CD31+ and the activation status was assessed by the

expression of HLA-DR+. Naïve CD8+ T-lymphocytes and naïve CD4+ T-lymphocytes were defined as CD62L+CD45RO-HLA-DR-. With the SCID-RTE tube, we identified patients with PID by low levels or absence of RTE in comparison to controls as well as low levels of naïve CD4+ and naïve CD8+ lymphocytes. These parameters yielded 100% sensitivity for SCID. All SCID patients had absence of RTE, including the patients with confirmed maternal engraftment or oligoclonally expanded T-cells characteristic for Omenn syndrome. Another dominant finding was the increased numbers of activated CD4+HLA-DR+ and CD8+HLA-DR+ lymphocytes. Therefore, the EuroFlow SCID-RTE tube together with the previously published PIDOT tube form a sensitive and complete cytometric diagnostic test suitable for patients suspected of severe PID (SCID or CID) as well as for children identified via newborn screening programs for SCID with low or absent T-cell receptor excision circles (TRECs).

Keywords: flow cytometric immunophenotyping, primary immunodeficiencies (PID), EuroFlow, standardization, severe combined immune deficiency (SCID), diagnosis

INTRODUCTION

Severe combined immunodeficiency (SCID) and combined immunodeficiency (CID) are two of the most severe forms of inherited disorders of the immune system (1, 2) with an incidence of 1:35,000–50,000 newborns. Patients are usually born asymptomatic, but they develop severe (opportunistic) infections, failure to thrive within the first months of life and generally die before the age of 1 year, unless they receive adequate and curative treatment. This includes hematopoietic stem cell transplantation (HSCT). For some genetic forms of SCID gene therapy is available (3, 4). HSCT is indicated immediately after birth, since patients transplanted before the age of 3.5 months or patients without infections have a superior prognosis as compared to those transplanted later or when infectious complications have accumulated (5, 6). In contrast, patients with CID usually do not have complete absence of T-lymphocytes as typically seen in SCID, but they frequently show profound impairment of T-cell immunity leading to severe infections, autoimmunity, and malignancies. Thus, the indication of HSCT for CID is less clear as it is less evident whether the T-cell deficiency is sufficiently severe to justify the risks of HSCT (7).

T-cells are generated in the thymus and released to peripheral blood as antigen inexperienced, naïve T-cells. These cells called "recent thymic emigrants" (RTE) are the recently formed naïve T-cells that are produced in the thymus and their numbers correlate with thymic output (8). To date, disease-causing mutations have been reported in 17 genes leading to SCID, and another 43 genes are reported as being mutated in CID (9, 10). The majority of SCID and CID patients presenting in the first 2 years of life have a defect in T-cell development in the thymus. A complete defect (null mutation) results in absence of T-cells, but hypomorphic ("leaky") mutations can give rise to an incomplete defect leading to presence of variable numbers of T-cells with poor immune function and inadequate control of autoreactivity. This leads to immunodeficiency and dysregulation such as seen in Omenn syndrome (11). Likewise, variable degree of T-cell immunodeficiency is found in patients diagnosed with 22q11.2 deletion syndrome (DiGeorge syndrome) (12).

An assay for early detection of SCID via newborn screening (NBS) has become available to identify T-cell lymphopenia directly after birth. This assay is based on measurement of T-cell receptor excision circles (TRECs) via quantitative PCR on dried blood spots (13). TRECs are formed as circular excision products during T-cell receptor gene rearrangement in developing T-cells in the thymus and are a molecular marker for recently formed T-lymphocytes. Absence or strongly reduced levels of TRECs are indicative for T-cell lymphopenia and can identify children who may have SCID. TRECs will not be detected in case of the presence of maternally engrafted T-cells. In addition, TRECs will also be low/absent in patients with Omenn Syndrome because of oligoclonal expansion of the autologous T-cells, which makes the TREC assay also useful in these subtypes of SCID. Follow-up diagnostic testing in case of low or absent TREC contents is needed to confirm the diagnosis by flow cytometric immunophenotyping and subsequently by targeted genetic testing for SCID-CID gene aberrations or broader genetic testing (e.g., WES or WGS) in combination with a SCID or PID filter. It should be noted that low or absent TRECs can also be identified in children with T-cell impairment syndromes [such as 22q11.2 deletion syndrome (14), Down's syndrome or Ataxia Telangiectasia], and children with T-cell impairment secondary to other neonatal conditions or patients with idiopathic lymphocytopenia (15, 16). Furthermore, low/absent TREC levels can also be found in preterm children or in children from mothers on immunosuppressive therapy (17).

Flow cytometric immunophenotyping of lymphocytes proved useful for the early diagnosis of SCID in patients with clinical symptoms or newborns with low/absent TRECs, showing complete lack of one or more lymphocyte lineages (T-cell, B-cell and NK cell) (15, 18). However, interpretation of this basic flow cytometric screening is not sufficient when T-cells are present, either due to a hypomorphic defect or due to the presence of maternal T-cell engraftment. Maternal T-cell engraftment is a relatively frequent finding in SCID (40% in a cohort of 121

patients from Ulm or 47% in the California cohort) (19, 20). In those cases, a flow cytometric test which allows more detailed phenotyping of the T-cells, including analysis of newly generated T-cells, is warranted. At present there is no consensus on the exact composition of a flow cytometric test, although critical parameters (Naïve T-cells, RTE, activated T-cells) are listed by the European Society for Immunodeficiencies (ESID), the American PID treatment consortium (PID-TC) and by other groups (7, 21).

Flow cytometry allows to discriminate naïve T-cell subsets from antigen experienced memory subsets by presence of typical markers (CD45RA isoform, costimulatory molecules CD27, homing receptor CCR7 and CD62L) and absence of memory markers (CD95 and CD45RO isoform) (22, 23). This is useful for diagnostic evaluation of patients with profound T-cell (function) deficiency, where T-cells are detectable (at normal or even increased levels) as a result of peripheral expansion of memory T-cell clones from either autologous or maternal origin. Immunophenotyping could show a skewed redistribution from naïve to memory and activated phenotypes within T-lymphocytes, which alerts for a possible lymphocyte development defect. Furthermore, newly generated T-cells released from the thymus to the periphery (RTE) can also be identified using flow cytometry. RTE are CD4+ T-cells with the highest TREC levels (24) and a phenotype characterized by expression of CD45RA and CD31 (25, 26). Finally, activated T-cells acquire a memory phenotype and temporary signs of activation, which can be detected via analysis of CD69, CD25, and HLA-DR, among other markers (27).

In this study, the EuroFlow PID group consortium has designed, developed and validated a standardized approach for flow cytometric evaluation of naïve, RTE and activated CD4+ and CD8+ T-cells that would offer a high sensitivity test toward disclosing (S)CID in line with the ESID diagnostic criteria in the settings of a multi-center collaboration study. The here developed 8 color "SCID-RTE tube" complements the recently published PIDOT tube (18, 28) for orientation and screening of primary immunodeficiencies (PID) of the lymphoid system. The combination of the two 8-color tubes (or a single 12-color variant of both tubes) could readily be applied in routine diagnostic screening for patients clinically suspected for having (S)CID, as well as in follow-up diagnostics in NBS programs.

MATERIALS AND METHODS

Patient and Control Samples

Our patient cohort consisted of 26 patients with a genetically defined PID diagnosed between birth and the age of 2 years at participating centers (**Table 1**). Genetic analysis was performed locally according to the routine procedures of the collaborating laboratories using Sanger sequencing or next generation sequencing (NGS). In addition, 44 healthy controls without any known hematological or immunological disorder in the same age range were also enrolled. The samples have been collected from 2013 to 2018. All 26 patient samples were collected according to the local medical ethics regulations of the participating centers, after informed consent was provided by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The study was approved by the local ethics committees of the participating centers: University of Salamanca, Salamanca, Spain (USAL-CSIC 20-02-2013); Charles University, Prague, Czech Republic (15-28541A); Erasmus MC, Rotterdam, The Netherlands (MEC-2013-026); University Hospital Ghent, Belgium (B670201629681/B670201214983) and St. Anne's University, Brno, Czech Republic (METC 1G2015)-.

SCID-RTE Tube Composition and Staining Protocol

The SCID-RTE tube aims to assess relevant lymphoid subpopulations important in PID diagnostics in a single 8 color test. It includes markers for T-cells (CD3, CD4, CD8, TCRγδ), including their naïve (CD62Lpos, CD45ROneg) and RTE (CD31pos) stages, as well as their activated forms (HLA-DR). Detailed composition and volumes of antibodies used are listed in **Table 2.**

The samples were processed in four EuroFlow laboratories (Charles University, Prague, Czech Republic; Erasmus MC, Rotterdam, The Netherlands; Ghent University, Ghent, Belgium; University of Salamanca, Salamanca, Spain) following standardized EuroFlow approaches (29, 30) (detailed protocols are publicly available at www.EuroFlow.org). In short, peripheral blood ($n = 66$) or cord blood ($n = 4$) (up to 2 ml) was mixed with ammonium chloride lysing solution (48 ml) and incubated for 15 min at room temperature in order to lyse erythrocytes. Obtained WBC were washed twice with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.09% sodium azide (NaN_3) and subsequently stained with the antibodies listed in **Table 2** in a final volume of 100 μl for 30 min at room temperature in the dark. Whenever possible, up to one million cells were processed, or all cells available in lymphopenic PID patients. In each case, at least 2×10^5 cells were stained. Next, the cells were incubated with 2 ml BD FACS™ Lysing Solution (BD Biosciences) for 10 min at room temperature in the dark, washed and resuspended in 250 μl washing solution.

Data Acquisition and Analysis

Data acquisition was performed on BD FACSCanto II, BD LSR II or BD FACSLyric instruments (BD Biosciences) equipped with 405, 488, and 633/640 nm lasers and PMT detectors, following the EuroFlow instrument set-up Standard Operating Protocol (29, 31). Data were analyzed using Infinicyt (Cytognos, Salamanca, Spain) and FlowJo (FlowJo LLC, Ashland, Oregon) software. Normal values for all T-cell subsets were determined as numbers above the 5th percentile of the healthy controls. In case of HLA-DR positive activated T-cells, we determined normal values as below the 95th percentile of the healthy controls (see **Table 3**). Therefore, specificity was by definition 95%. For sensitivity calculations we divided the number of patients with an abnormal value by the total number of patients measured in each parameter/subset separately (see **Table 3**). For statistical analysis, GraphPad Prism software Mann-Whitney test was used.

TABLE 1 | Characteristics of patients, WBC and lymphocytes subsets (TBNK) reported by the referring clinician (x 10e3/μl).

Category	Case no.	Gender	Disease	Mutation	Protein	WBC	T-cells (abs)	B-cells (abs)	NK-cells (abs)	Age
SCID	Case_1	F	SCID, CD3E deficiency	CD3E exon 6 c.173delT	p.Leu58HisfsX9	13.9	0.32	2.6	1.4	0.3
SCID	Case_2	M	SCID, ADA deficiency	ADA exon 4 homozygous c.302G > A	p.Arg101Gln	2.5	0.03	0	0.01	1.3
SCID	Case_3	M	SCID, JAK3 deficiency with mat.engr.	JAK3 heterozygous c.561delT, c.2066C > T	p.Val188SerfsX14, p.Pro689Leu	11.8	6.31	3.02	0.02	1.8
SCID	Case_4	F	SCID, JAK3 deficiency	JAK3 exon 12 homozygoot c.1765G > A (NM_000215)	p.Gly589Ser	6.5	0.03	0.8	0.08	0
SCID	Case_5	F	SCID, JAK3 deficiency with mat.engr.	JAK3 exon 5 c.578G > A, exon 19 c.2712C > A	p.Cys193Tyr	15.5	1.68	0.37	0.1	0.2
SCID	Case_6	M	SCID, Cernunnos/XLF deficiency	NHEJ1 exon 5 homozygoot c.532C > T	p.Arg178X	3.4	0.22	0.04	0	0.9
SCID	Case_7	F	SCID, Artemis deficiency	DCLRE1C c.1A > C c.401C > G (compound heterozygote)	M1V, T134R (Met1Val, Thr134Arg)	3.6	0.00132	0	0.0018	0.4
SCID	Case_8	M	SCID, RAG2 deficiency	RAG2 homozygous c.1280_1281insTGGATAT	p.Asn428GlyfsX12	33.1	0.04	0.01	1.61	0.2
SCID	Case_9	M	SCID, RAG2 deficiency	RAG2 c.107G > A	p.Trp36*	2.8	0.04236	0.0444	0.0678	0.2
SCID	Case_10	M	Omenn syndrome, RAG1 deficiency	RAG1 c.983G > A/c.1186C > T (compound heterozygote)	pCys328Tyr/pArg396Cys	27.9	6.767	0.0303	1.818	0
SCID	Case_11	M	Omenn syndrome, RAG1 deficiency	RAG1 exon 2 c.519del	p.Glu174Serfs*27	8.05	2.15	0	0.429	0.3
SCID	Case_12	M	SCID, IL2RG deficiency with mat.engr.	IL2RG c.270-1G > A	n.d.	3.6	0.04	0.57	0	0.7
SCID	Case_13	M	SCID, IL2RG deficiency	IL2RG c.613G > A	p. Trp174*	9.5	0.001045	0.22325	0.022895	0.7
SCID	Case_14	M	SCID, IL2RG deficiency with mat.engr.	IL2RG c.269+3A > T	n.d.	5.4	0.6804	0.783	0.00891	0.5
SCID	Case_15	M	SCID, IL2RG deficiency	IL2RG exon 5 hemizygoot c.595-1G > T	n.d.	8.6	0	0.6	0.02	0.3
other PID	Case_16	M	CID, PNP deficiency	PNP c.700C > T	p.Arg234X	6.1	0.5	0.07	0.01	1.6
other PID	Case_17	M	ZAP70 deficiency	ZAP70 exon 10 homozygoot c.1193C > T	p.Ile398Ser	11.3	2.18	1.03	0.17	0.6
other PID	Case_18	M	Wiskott-Aldrich syndrome	WAS c. 1271_1295del	p.Gly424Glufs*13	8.4	1.47	0.95	0.25	0.3
other PID	Case_19	M	Wiskott-Aldrich syndrome	WAS c.344A > G	p.His115Arg	4	1.271	0.4305	0.3075	1.1
other PID	Case_20	M	Complete DiGeorge syndorme	del22q11.2		6.7	0.48776	0.003819	0.1206	1.6
other PID	Case_21	F	Complete DiGeorge syndorme	del22q11.2		5.1	0.00126684	0.655	0.504	0.2
other PID	Case_22	F	DiGeorge syndrome	del22q11.2		9.9	0.914354	1.342852	1.008826	0.6
other PID	Case_23	M	DiGeorge syndrome	del22q11.2		6.7	0.97	1.29	0.7	0.3
other PID	Case_24	M	Takenouchi-Kosaki syndrome	CDC42 c.191A > G	p.Tyr64Cys	2.4	0.436	0.094	0.094	1.5
other PID	Case_25	M	IPEX syndrome	FOXP3 c.721T > C	S241P (p.Ser241Pro)	15.135	2.42353	1.013115	0.349624	0.2
other PID	Case_26	M	Autoimmune lymphoproliferative sy	FAS exon 7 heterozygous (frameshift)	n.d.	29.8	20.818	2.146	0.226	0.3

TABLE 2 | Composition of the EuroFlow SCID-RTE tube*.

Marker	Fluorochrome	Clone	Source	Catalog number	µl/test
CD3	APC	SK7	BD Biosciences	345767	2.5
CD4	BV510	OKT4	Biolegend	317443	1.5
CD8	APC-Alexa750	B9.11	Beckman Coulter	A94683	1.5
CD31	PE	MEM-05	Exbio	1P-273-T100	5
CD45RO	FITC	UCHL1	Exbio	1F-498-T100	10
CD62L	BV421	DREG-56	Biolegend	304827	2
HLA-DR	PerCP-Cy5.5	L243	Biolegend	307629	1.5
TCRγδ	PE-Cy7	11F2	BD Biosciences	649806	2.5

*Both the SCID-RTE tube and the PIDOT tube have originally be designed for application in 8-color format. However, because of their strong complementarity, it can be efficient and cost-effective to use a 12-color "combined PIDOT & SCID-RTE variant" by supplementing the PIDOT tube with the CD45RO, CD31, HLA-DR, and CD62L markers.

RESULTS

Composition of the SCID-RTE Tube

The SCID-RTE tube was designed with the purpose of identifying the relevant lymphoid subpopulations important in PID diagnostics of severe PID in newborns, using a single 8-color test. It identifies naïve CD4+ T cells and among them, the RTEs. The definition of naïve T-cells includes a selection of non-activated (HLA-DR negative) cells, together with absence of CD45RO (a memory T-cell marker) and the presence of the naïve T-cell marker L-selectin (CD62L). The definition of RTE uses the naïve T-cell gate and is further complemented by CD31, Platelet endothelial cell adhesion molecule (PECAM-1) (**Table 2**, **Figure 1**). After gating T-cells as CD3+ and lymphocytes on FSC and SSC, four markers (CD3, TCRγδ, CD4, and CD8) were used to define TCRγδ+ and TCRγδ- CD4+, CD8+ and double negative (DN) T-cells (**Figure 1A**, **Supplemental Figure 1**). The CD4+ T-cells were further subdivided into RTE, naïve, central memory (CM), effector memory CD45RO+ (EMRO+) and CD45RO- (EMRO-) and activated memory T-cells (**Figures 1B,D**); for CD8+ T-cells the same subsets were defined except for the RTEs (**Figures 1C,D**). The total set and hierarchy of T-cell subsets that was identified is listed in **Figure 1D**. To offer intuitive and fast interpretation of the complete lymphoid compartment we developed a new analysis and visualization strategy for the SCID-RTE tube using principle component analysis (PCA)-based multidimensional views (APS graphs). First, reference plots were generated using a set of 10 samples of healthy donors in Infinicyt software. The lymphocyte populations were manually analyzed and subsequently, the most discriminating projection into a single APS graph was determined (**Figure 1E**). A software tool for automated identification of the cell populations present in the SCID/RTE tube was built, containing normal blood samples stained with the same antibody combination.

Identification of RTEs by the SCID-RTE Tube in PID Patients

The SCID-RTE tube allows analysis of the naïve and memory subsets of T-cells (**Figure 2A**) that are abnormally distributed in patients with PID (**Figure 2B**). Typically, their numbers are different in childhood compared to the adult age (**Figure 3**), however within 2 years of age a general threshold for CD4+ RTE (<800 cell/µl), naïve CD4+ (<1,000 cell/µl) and naïve CD8+ lymphocytes (<290 cell/µl) is justified. Notably, the number of RTEs are abundant in childhood, whereas the numbers of activated memory CD4+ and CD8+ T-cells are low (**Figures 2A, 3**).

SCID patients that present without T-cells (**Figure 2B**, *IL2RG*) are straightforwardly identified by the SCID-RTE tube, but they never pose a diagnostic dilemma even in a simple T-B-NK flow cytometric assay. However, in SCID and Omenn syndrome patients with paradoxically normal or even increased total absolute numbers of T-cells, the SCID-RTE tube allows detection of the activation status of T-cells (HLA-DR positive). These activated T-cells can be of maternal origin in SCID with maternal engraftment (**Figure 2B**, *JAK3 with maternal engraftment*) or can be oligoclonal, expanded T-cells in Omenn syndrome patients (**Figure 2B**, *RAG1 Omenn syndrome*). RTE cells are virtually absent in these patients with T-cell production defects (SCID and Omenn patients). Patients with PNP deficiency and complete DiGeorge syndrome also lack RTEs, but patients with ZAP70 deficiency, Wiskott-Aldrich Syndrome and ALPS have detectable RTEs (**Figure 2B,** detailed dot plots shown in **Supplemental Figures 2**, **3**).

Absence of RTEs in SCID Patients

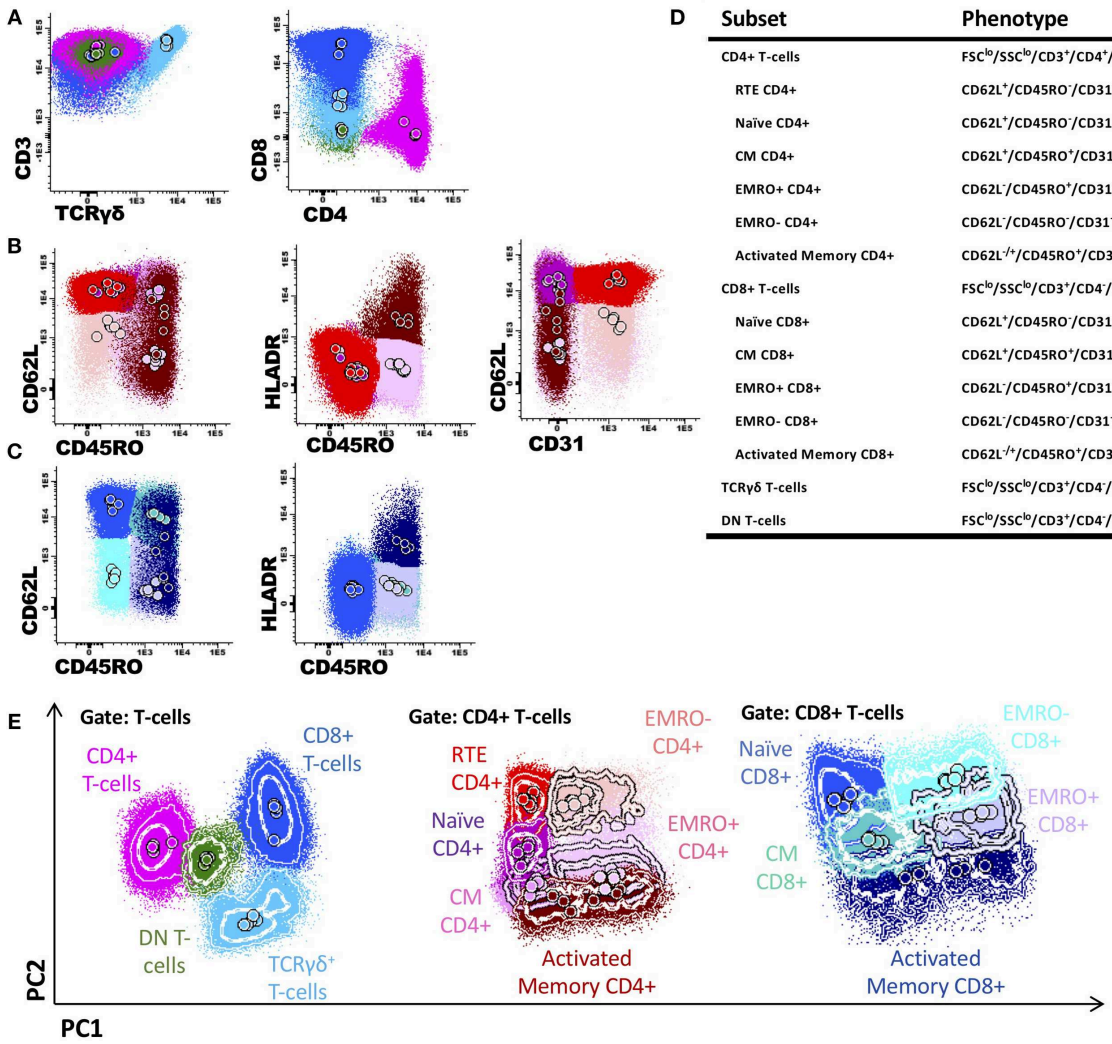
In our current study, we analyzed 15 SCID patients and 11 other PID patients diagnosed before 2 years of age. SCID patients had disease-causing mutations in *RAG1 or RAG2* ($n = 4$, two of them presented with Omenn syndrome), IL2RG ($n = 4$, one of them with confirmed maternal engraftment), *NHEJ1* ($n = 1$), *CD3E* ($n = 1$), *ADA* ($n = 1$), *JAK3* ($n = 3$, two of them with maternal engraftment) and *DCLRE1C* ($n = 1$) (see **Table 2** and **Supplemental Figure 2**).

In the SCID patients, the absolute levels of CD3+ T-cells were strongly reduced ($n = 7$) or undetectable ($n = 5$), but for three patients (20% of our cohort) the absolute CD3+ T-cell counts were in the normal range (see **Table 3**). These patients were proven to have maternal engrafted T-cells ($n = 2$) or oligoclonally expanded cells characteristic for Omenn syndrome ($n = 1$).

TABLE 3 | Lymphocytes subsets evaluated by SCID-RTE tube.

Patient code	Disease	Category	CD3+ of Lym	CD3+ abs	TCRgd+ of T	TCRgd+ abs	CD4+ T of T	CD4+T abs	CD8+ T of T	CD8+T abs	CD4+RTE of CD4	CD4+RTE abs	Naive CD4+ of CD4	Naive CD4+ abs	Naive CD8+ of CD8	Naive CD8+ abs	HLA-DR+ of CD4	HLA-DR+ CD4+ abs	HLA-DR+ of CD8	HLA-DR+ CD8+ abs
Case_1	CD3E	SCID	**7**	**246**	0	0	**5**	**13**	93	**228**	**0**	**0**	**0**	**0**	**2**	**5**	**93**	12	**83**	**188**
Case_2	ADA	SCID	55	**18**	**13**	2	**1**	**0**	83	**15**	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**	**n/a**	**93**	14
Case_3	JAK3	SCID	69	5,610	1	67	**4**	**210**	94	5,256	**0**	**0**	**0**	**1**	**2**	**117**	**64**	**134**	**95**	**4,967**
Case_4	JAK3	SCID	**2**	**15**	3	0	**1**	**0**	85	**12**	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**	**n/a**	**99**	12
Case_5	JAK3	SCID	80	2,247	0	4	98	2,202	**1**	**23**	**0**	**0**	**0**	**1**	**0**	**0**	**86**	**1,896**	**86**	19
Case_6	XLF	SCID	**38**	**294**	**89**	263	**0**	**1**	**2**	**7**	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**	**n/a**	**64**	5
Case_7	Artemis	SCID	**18**	**84**	**55**	46	**38**	**31**	**1**	**1**	**6**	**2**	**6**	**2**	**31**	**0**	**88**	28	**44**	0
Case_8	RAG2	SCID	**2**	**40**	1	1	71	**29**	**1**	**0**	**0**	**0**	**0**	**0**	**17**	**0**	**91**	26	**n/a**	**n/a**
Case_9	RAG2	SCID	**32**	**78**	5	4	86	**67**	**5**	**4**	**0**	**0**	**0**	**0**	**3**	**0**	**66**	44	**77**	3
Case_10	RAG1	SCID	68	4,621	3	158	**43**	1,964	44	2,024	**0**	**0**	**0**	**0**	**0**	**1**	**85**	**1,661**	**86**	**1,739**
Case_11	RAG1	SCID	69	**1,916**	6	109	88	1,690	**3**	**64**	**0**	**0**	**0**	**2**	**1**	**1**	**68**	**1,154**	**83**	53
Case_12	IL2RG	SCID	**12**	**92**	0	0	71	**65**	28	**25**	**0**	**0**	**0**	**0**	**4**	**1**	**94**	61	**81**	20
Case_13	IL2RG	SCID	**0**	**1**	5	0	**31**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**	**n/a**	**n/a**	**n/a**
Case_14	IL2RG	SCID	**45**	**677**	**25**	171	**48**	**325**	23	**156**	**0**	**0**	**1**	**3**	**7**	**11**	**87**	**282**	**94**	**147**
Case_15	IL2RG	SCID	**0**	**1**	**17**	0	58	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**	**n/a**	**n/a**	**n/a**
Case_16	PNP	other PID	63	**281**	3	7	**48**	**134**	37	**105**	**0**	**0**	**0**	**0**	**0**	**0**	**63**	**84**	**99**	**103**
Case_17	ZAP70	other PID	**51**	**1,661**	3	47	89	1,482	**2**	**29**	**24**	**357**	**34**	**505**	**19**	**5**	**17**	**246**	**30**	9
Case_18	WAS	other PID	**50**	**1,092**	4	41	82	**891**	**13**	**138**	**53**	**470**	82	**730**	**56**	**78**	3	23	3	4
Case_19	WAS	other PID	57	**978**	**28**	271	**31**	**303**	38	376	**18**	**55**	**40**	**122**	**6**	**23**	**39**	**119**	**85**	**319**
Case_20	del22q11.2	other PID	**50**	**328**	**37**	122	**15**	**47**	**3**	**9**	**0**	**0**	**0**	**0**	**1**	**0**	**43**	21	**65**	6
Case_21	del22q11.2	other PID	**0**	**1**	**10**	0	**0**	**0**	70	**1**	**0**	**0**	**0**	**0**	**14**	**0**	**n/a**	**n/a**	**58**	1
Case_22	del22q11.2	other PID	**27**	**1,592**	**9**	138	65	**1,030**	22	344	**56**	**579**	**71**	**729**	87	300	**5**	56	4	14
Case_23	del22q11.2	other PID	**33**	**756**	**8**	63	65	**493**	22	**169**	**50**	**246**	72	**356**	87	**147**	**4**	20	4	7
Case_24	CDC42	other PID	63	**465**	**39**	180	**34**	**159**	23	**107**	**40**	**63**	**66**	**105**	**58**	**63**	**6**	10	**21**	22
Case_25	FOXP3	other PID	61	**1,664**	1	22	70	**1,157**	22	363	**40**	**465**	**66**	**765**	81	295	**4**	48	5	19
Case_26	FAS	other PID	86	12,833	3	**444**	**26**	3,324	15	1,912	58	1,938	81	2,692	92	1,761	**11**	**356**	5	**88**
Controls																				
5th percentile			**52**	**2,010**	0.7	20	**54**	**1,201**	**13**	**335**	**58**	**840**	**72**	**1,030**	**60**	**287**	0	9	0	2
95th percentile			86	6,626	**7**	**340**	82	4,094	35	2,204	86	2,393	96	3,149	96	1,614	**3**	**66**	**11**	**80**
Sensitivity other PID			55%	91%	55%	20%	55%	82%	27%	64%	91%	91%	73%	91%	64%	73%	90%	40%	55%	27%
Sensitivity SCID			67%	80%	33%	60%	60%	80%	53%	87%	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	50%	**100%**	33%

Absolute counts (abs) as 10e3/μl. Values outside the normal range are in bold. Range obtained in controls (5th and 95th percentile) is given below the table. Sensitivity to disclose abnormal values in other PID and SCID group is given for each measurement in the bottom two rows. Most informative parameters are highlighted in gray.



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Subset	Phenotype
CD4+ T-cells	$FSC^{lo}/SSC^{lo}/CD3^{+}/CD4^{+}/CD8^{-}/TCR\gamma\delta^{-}$
RTE CD4+	$CD62L^{+}/CD45RO^{-}/CD31^{+}/HLA\text{-}DR^{-}$
Naïve CD4+	$CD62L^{+}/CD45RO^{-}/CD31^{-}/HLA\text{-}DR^{-}$
CM CD4+	$CD62L^{+}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{-}$
EMRO+ CD4+	$CD62L^{-}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{-}$
EMRO- CD4+	$CD62L^{-}/CD45RO^{-}/CD31^{-/+}/HLA\text{-}DR^{-}$
Activated Memory CD4+	$CD62L^{-/+}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{+}$
CD8+ T-cells	$FSC^{lo}/SSC^{lo}/CD3^{+}/CD4^{-}/CD8^{+}/TCR\gamma\delta^{-}$
Naïve CD8+	$CD62L^{+}/CD45RO^{-}/CD31^{-/+}/HLA\text{-}DR^{-}$
CM CD8+	$CD62L^{+}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{-}$
EMRO+ CD8+	$CD62L^{-}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{-}$
EMRO- CD8+	$CD62L^{-}/CD45RO^{-}/CD31^{-/+}/HLA\text{-}DR^{-}$
Activated Memory CD8+	$CD62L^{-/+}/CD45RO^{+}/CD31^{-/+}/HLA\text{-}DR^{+}$
TCRγδ T-cells	$FSC^{lo}/SSC^{lo}/CD3^{+}/CD4^{-}/CD8^{-/+}/TCR\gamma\delta^{+}$
DN T-cells	$FSC^{lo}/SSC^{lo}/CD3^{+}/CD4^{-}/CD8^{-}/TCR\gamma\delta^{-}$

FIGURE 1 | Gating T-cell subsets and generation of a reference principal component analysis representation in an n-dimensional space for SCID-RTE tube. **(A)** After gating T-cells as CD3+ and FSC^{lo} and SSC^{lo}, the markers TCRγδ+, in combination with CD4 and CD8 were used to define TCRγδ+ T-cells (light blue), CD4+CD8-TCRγδ- T-cells (pink); CD4-CD8+TCRγδ- T-cells (dark blue) and CD4-CD8- TCRγδ- double negative T-cells (green). **(B)** The CD4+ T-cell subsets were further subdivided into recent thymic emigrants (RTE; CD62L+CD45RO-HLDR-CD31+; red), naïve (CD62L+CD45RO-HLDR-CD31-; purple), central memory (CM; CD62L+CD45RO+HLDR-; orchid), effector memory CD45RO+ (EMRO+; CD62L-CD45RO+HLDR-; mauve), effector memory CD45RO- (EMRO-; CD62L-CD45RO-HLDR-; pink) and activated memory (CD45RO+HLDR+; burgundy) CD4+ T cells. **(C)** The CD8+ T-cell maturation subsets were further subdivided into naïve (CD62L+CD45RO-HLDR-; blue), central memory (CM; CD62L+CD45RO+HLDR-; blue-green), effector memory CD45RO+ (EMRO+; CD62L-CD45RO+HLDR-; periwinkle blue), effector memory CD45RO- (EMRO-; CD62L-CD45RO-HLDR-; cyan) and activated memory (CD45RO+HLDR+; navy blue) CD8+ T cells. **(D)** Definition and hierarchy of the defined subsets. **(E)** Principal component analysis representation (APS view) based on the most discriminating parameters for T-cell populations, and CD4+ T-cells and CD8+ T-cell subsets.

Application of the SCID-RTE tube showed that all SCID patients completely lacked RTE cells and other forms of naïve CD4+ and CD8+ T cells (**Figure 4A**), even the patients with normal T-cell counts (due to maternal T-cells or oligoclonal expansion). The T-cells that could be detected had signs of massive activation (64–94% HLA-DR+ in CD4+ and 44–99 % in CD8+ T cells) (**Figure 4B**). Overall, the SCID-RTE tube detected severely decreased or absent numbers of RTE and naïve CD4+ and CD8+ T-cell subsets in all SCID patients. In the SCID patients with detectable levels of T-cells, the phenotype was characterized by activation (HLA-DR+) and had a memory phenotype (CD45RO+).

RTEs in Other Severe PID Diagnosed Before 2 Years of Age

Patients diagnosed with other severe forms of PID (Other PID, $n = 11$) had diverse molecular defects [*ZAP70* ($n = 1$), *WAS* ($n = 2$), *PNP* ($n = 1$), *FOXP3* ($n = 1$) del22q11.2 (DiGeorge $n = 2$; complete DiGeorge $n = 2$), *CDC42* ($n = 1$) and *FAS* ($n = 1$)] (see **Table 2** and **Supplemental Figure 3**). Except for the patient

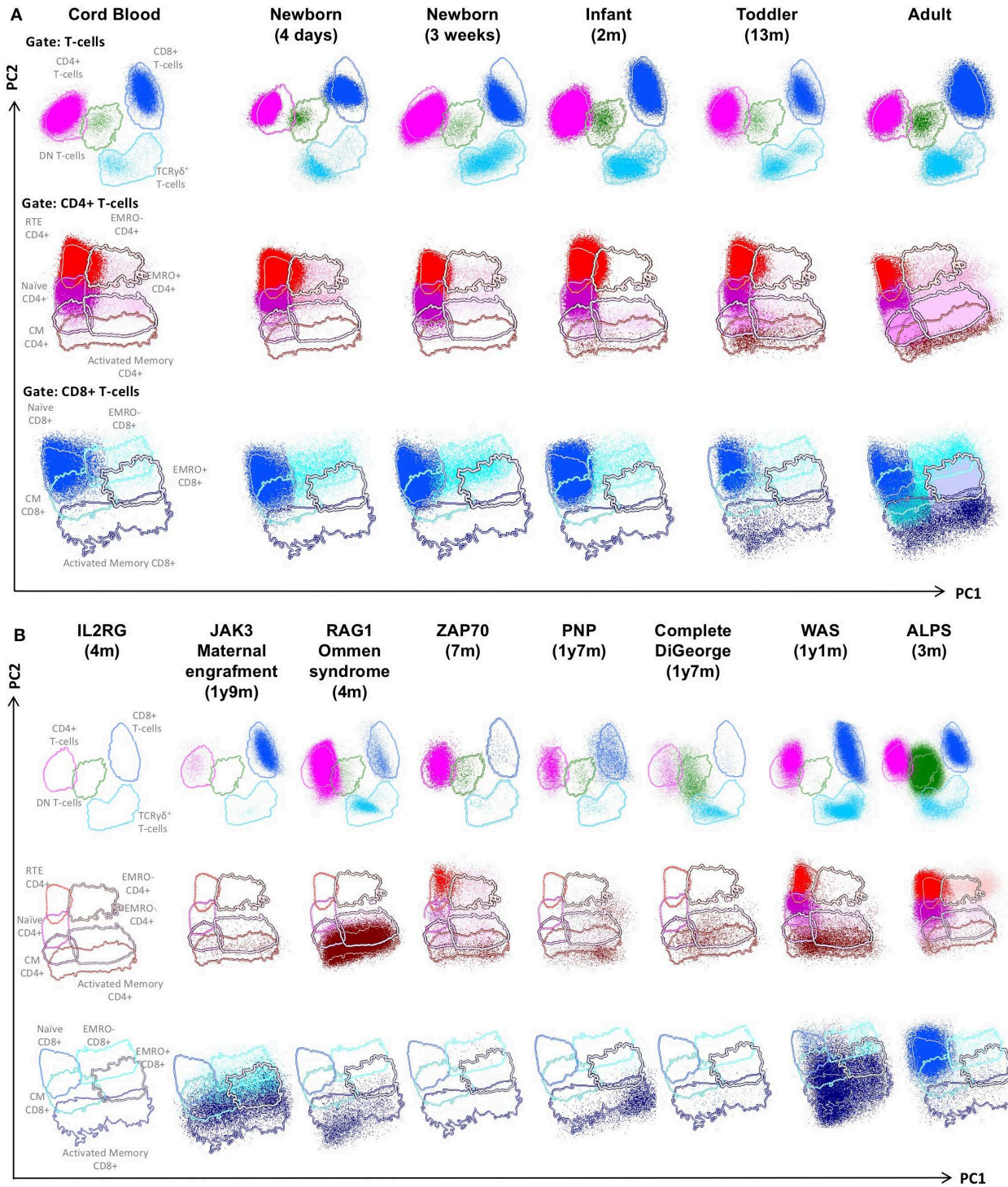

FIGURE 2 | PCA representation of SCID-RTE tube results, showing the distinct blood T-cell subsets in the supervised PCA analysis of blood samples from healthy donors of different age **(A)** and SCID and CID patients **(B)**. From the top down, APS plots of gated total, CD4+ and CD8+ T-cells are shown. Lines depict a 2 standard deviation boundary of all controls combined. **(A)** PCA (APS views) of all T-cell subsets of cord blood and peripheral blood from donors of different age. **(B)** PCA (APS views) of all T-cell subsets of the following patients: a IL2RG-deficient patient, a JAK3-deficient patient with maternal engraftment, a RAG1-deficient Omenn syndrome, a ZAP70-deficient patient, a PNP-deficient patient, a complete DiGeorge syndrome, a Wiskott-Aldrich syndrome (WAS) and an autoimmune lymphoproliferative syndrome (ALPS) patient.

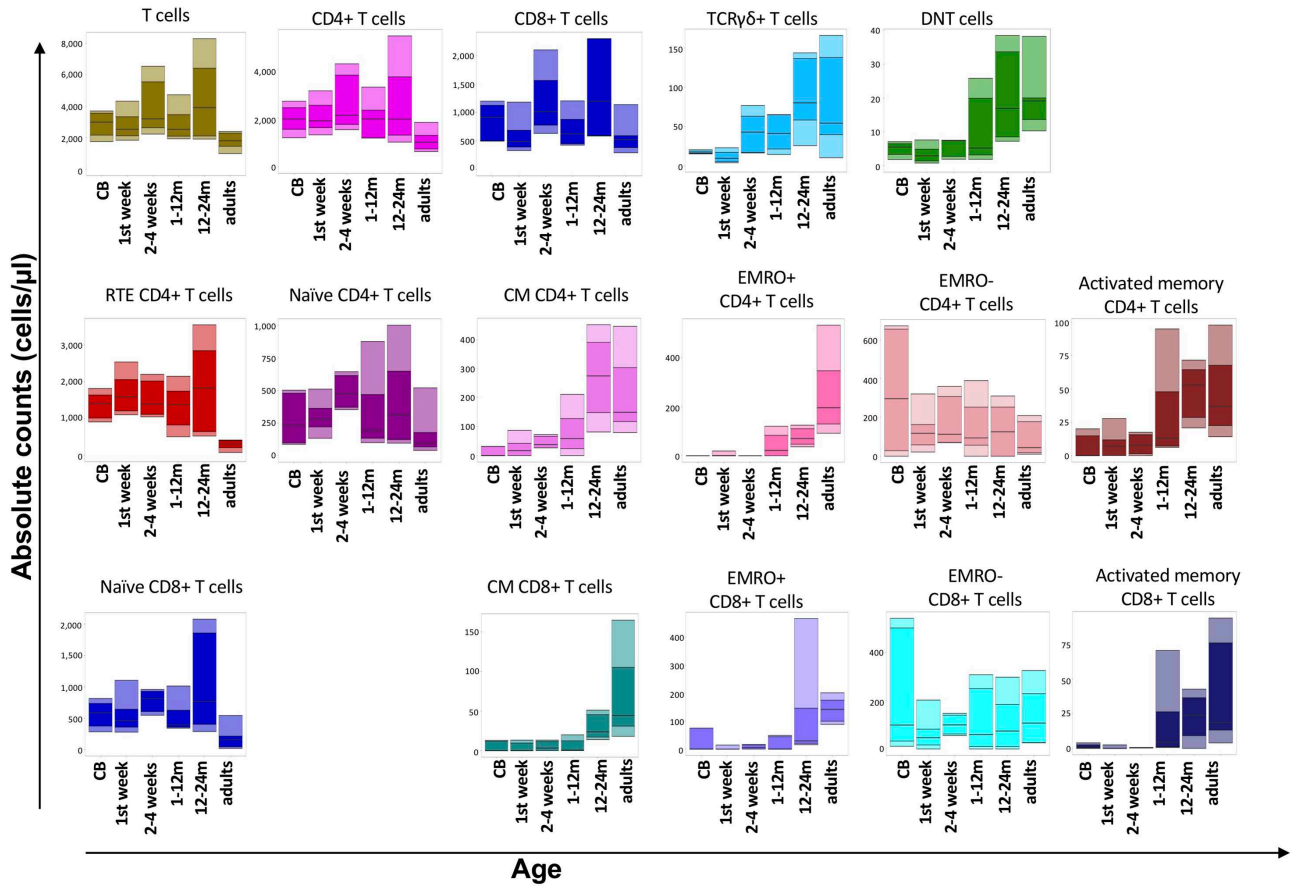

FIGURE 3 | Flow cytometric analysis of T-cell populations using the EuroFlow SCID/RTE tube in 56 healthy controls of five different age ranges. All values of this reference data set are displayed as bar graphs representing the median, and p10, p25, p75, and p90 percentiles. For data visualization package gplot2 for the statistical language R was used.

with autoimmune lymphoproliferative syndrome (ALPS) due to FAS mutation, all had decreased absolute counts of CD3+ T-cells compared to controls. However, only one complete DiGeorge patient had <300 T-cells/µl in the conventional TBNK test, which is considered a diagnostic threshold in SCID patients.

When assessed by the SCID-RTE tube the patients showed a heterogeneous pattern of T-cell subset abnormalities ranging from strongly reduced/absent to normal numbers. However, all of the patients showed at least one abnormality. With the exception of the ALPS patient who presented with normal proportion of naïve CD4+ T-cells (and elevated naïve CD8+ T-cell counts) (32), all had reduced naïve CD4+ T-cells and RTEs below 5th percentile of healthy (**Figures 2B**, **4A**, **Table 3**). The ALPS patient was characterized by massively increased T-cells especially of double negative T-cells (56% of CD3+TCRγδ- cells) and activated CD4+ T-cells (11%). As previously described (33), we found a high frequency of TCRγδ+ T cells (9–39 % of CD3+ cells), in patients with Wiskott-Aldrich syndrome (WAS) as well as in DiGeorge ($n = 2$) and complete DiGeorge patients. We also identified a high frequency of TCRγδ+ T-cells in CDC42 deficiency. Four had normal total CD8+ T-cell counts, but reduced naïve CD8+ T-cells and showed signs of activation: WAS, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), DiGeorge (**Table 3**).

On top of reduction or absence of naïve and RTE subsets of CD4+ T-cells (**Table 3**), three of the four DiGeorge patients showed decreased CD8+ T cells and their naïve subsets. Activation of T cells (as measured by HLA-DR+) ranged from mild to high. Both patients with WAS had profoundly reduced naïve CD8+ T-cells, reduced naïve CD4+ T-cells and RTE, activation was found in both CD4+ and CD8+ T cells in one of the WAS patients. The complete dataset of findings for all patients is provided in **Table 3**.

Added Value of SCID-RTE on Top of the PIDOT Tube

The proposed SCID-RTE tube is both a confirmation and extension of the PIDOT tube. As the reduction of naïve T-cells was one of the most important hallmarks of (S)CID and PID in our cohort, as well as in the large group of PID patients published previously (18), we investigated whether the definition of naïve T-cells in the PIDOT tube (CD45RA+CD27+) corresponds to the SCID-RTE tube

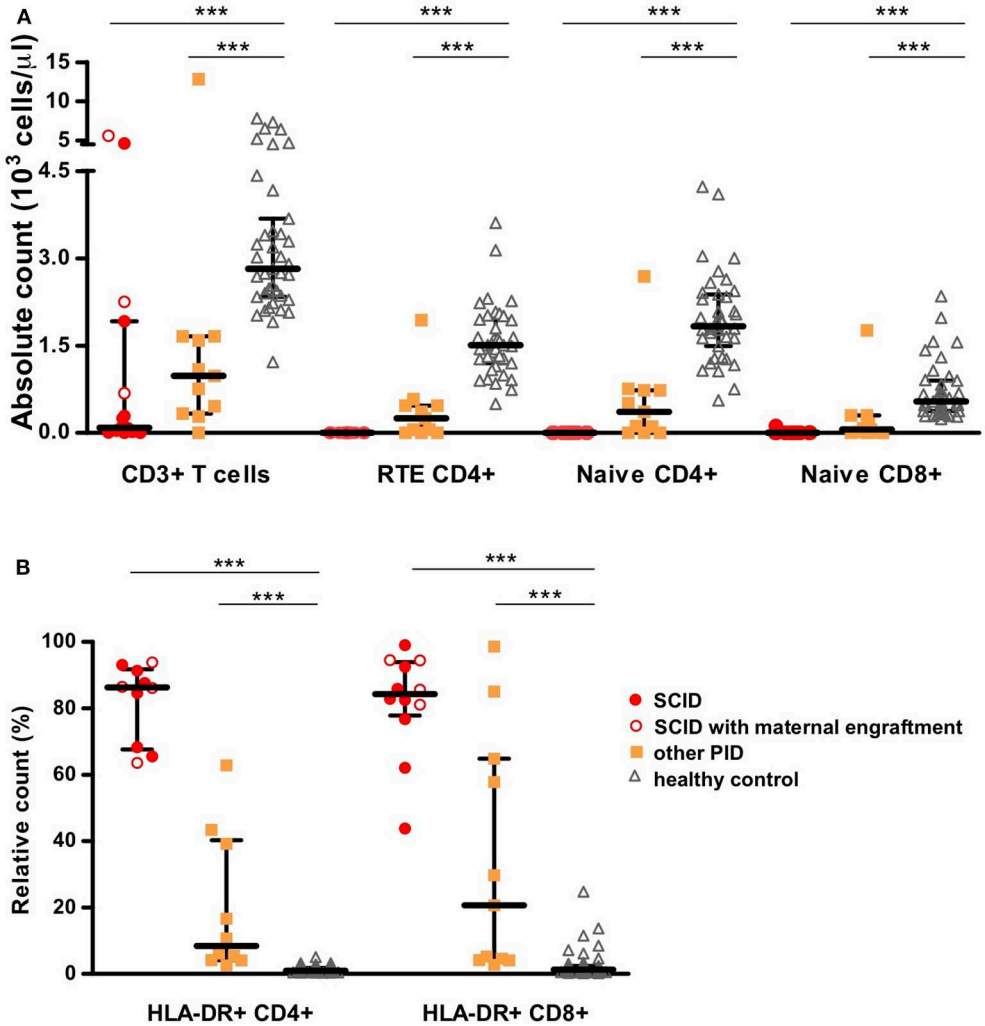

FIGURE 4 | T-cell subset counts determined using the EuroFlow SCID-RTE tube. **(A)** Absolute values of CD3+ T-cells, RTE CD4+ cells, naïve CD4+ or CD8+ T-cells. **(B)** Relative values of activated T-cells based on the expression of HLA-DR molecule on CD4+ T-cells (HLA-DR+CD4+) or CD8+ T-cells (HLA-DR+CD4+). SCID patients ($n = 15$) are represented as red circles where open circles show patients with maternal engraftment, other PID patients ($n = 11$) as orange squares and healthy controls ($n = 44$) as gray triangles. Detailed gating strategy is shown in **Supplemental Figure 1**. ***$P \leq 0.0005$.

definition (CD45ROnegCD62L+HLA-DRneg). Indeed, we found that both approaches yield correlating values in the PID patients (**Figure 5**). Thus, the PIDOT tube is capable of detecting reduction of naïve T-cells and directing the testing toward confirmation with the SCID-RTE tube, which also allows the specific detection of RTEs and activation status, which is particularly important in patients with normal or close to normal T-cell counts.

Both the PIDOT tube and the SCID-RTE tube have originally been designed for application in an 8-color format. However, because of their strong complementarity, it can be efficient and cost-effective to use the 12-color combined PIDOT & SCID-RTE variant in cases suspicious of (S)CID, by supplementing the PIDOT tube with the CD45RO, CD31, HLA-DR, and CD62L markers.

Specificity and Sensitivity

Next, we determined the sensitivity of the SCID-RTE tube to find abnormal values (defined as below the 5th percentile, thus allowing for specificity 95%) in our cohort of 15 (S)CID and 11 other PID patients.

The sensitivity of values of naïve CD4+, CD8+, and CD4+ RTE yielded 100% sensitivity to detect (S)CID (see **Table 3**). This was despite the fact that several (S)CID patients presented with close to normal total T-cells and their basic subsets (CD4+ and CD8+ T-cells). Whenever T-cells were detectable, their activation status (percentage of HLA-DR) was an equally sensitive marker for (S)CID, as the naïve T-cells and RTEs.

However, naïve T-cells and RTEs were also abnormal in the group of other PID, implying that these patients could also be recognized and referred for genetic testing. Thus, it can be

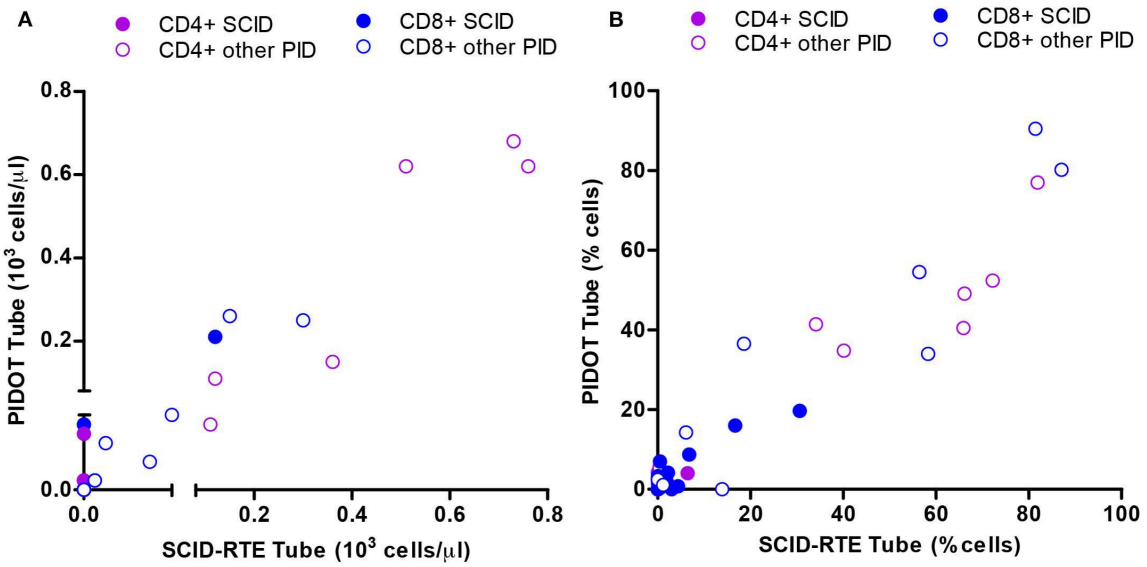

FIGURE 5 | Correlation between the SCID-RTE and the PIDOT tube in determining the levels of naïve CD4+ and CD8+ T-cell subsets in PID patients. **(A)** Absolute counts or **(B)** Relative counts for each PID patient ($n = 24$) of naïve CD4+ T-cells (blue) and naïve CD8+ T-cells (green) measured by the SCID-RTE tube (x axis) and the PIDOT tube (y axis). SCID (closed circles), other PID (open circles) patients. Detailed gating strategy is shown in **Supplemental Figure 1**.

concluded that in case of reduced naïve T-cells and RTEs fast genetic testing is urgently needed.

DISCUSSION

There have been multiple initiatives for establishing comprehensive and detailed reference values of human lymphocyte subsets in children by several groups (34–36). These studies were done by using two to four color flow cytometry. There was no attempt to standardization and no clear indication about the utility of individual subsets' abnormalities for PID diagnostics. Recently, Takashima et al. reported on a detailed set of seven 8–10 color flow cytometry panels used to investigate 75 PID patients, where they also found lack of naïve T-cells in SCID (also with maternal engraftment), Ataxia Telangiectasia and CMCD (37). However, this 7-tube approach would be demanding to use at large scale, and it requires relatively high sample volume for the seven tube-aliquots. With the EuroFlow PID consortium, we developed a standardized approach for flow cytometry testing in PID (38), which includes a tube for screening and orientation (PIDOT) (18), two 8-color tubes for analysis of pre- and post-germinal center B-cells, and an additional isotype tube allowing full characterization of B-cells, including analysis of IgH isotype and subclass distribution within the memory B-cell (MBC) and plasma cell (PCs) compartments (39). The EuroFlow approach offers a systematic approach to diagnostics with a modular design (37).

Here we focused on the feasibility and performance characteristics of the EuroFlow SCID-RTE tube for diagnostic use in (S)CID and severe PID in a cohort of 26 patients, genetically diagnosed before the age of 2 years. The challenge in revealing SCID patients comes from the fact that a large portion of them presents with detectable T-cells that are either autologous oligoclonal T-cells as seen in Omenn syndrome (40, 41), or arise from maternal engraftment [40% of SCID according to Mueller et al. (19)]. The EuroFlow SCID-RTE tube overcomes the limitations of the basic T, B, NK test that cannot evaluate the nature of the T-cell subsets. In particular, the CD4+ naïve, CD8+ naïve, and CD4+ RTE subsets are shown to be decreased in all SCID and a great majority of other PID in our cohort. The RTE subset was reported as a useful proxy for thymic output measurements (25, 42–45), correlating to TREC levels (43, 45–47), that are used in newborn screening programs for SCID.

Since (S)CID patients harbor deleterious mutations that prevent normal T-cell development, any T-cells in their bloodstream must be expanded T-cells of either autologous oligoclonal origin or maternal origin. In order to improve the RTE definition for patients with putative peripheral expansion of T-cells, we gated not only on CD31+CD45RO-CD4+ T-cells, but we additionally excluded TCRγδ+ and HLA-DR positive cells and restricted the gate to include CD62L positive cells only. Thus, only naïve, non-activated CD4+ T-cells (non-TCR γδ+) are counted as RTE. This improved the accuracy of the RTE measurements, particularly in other PID patients with massive presence of HLA-DR, where some activated (HLA-DR+) cells would be otherwise considered RTE. HLA-DR was reported as a marker of residual T-cells in Omenn syndrome patients by Saint Basil et al. (27) already in 1991. The biological significance and mode of HLA-DR acquisition by T-cells is thought to be explained by acquisition of the molecule from antigen presenting cell (APC) after T-cell-APC contact (48).

A difficult PID category to be diagnosed with the SCID-RTE tube would be DiGeorge syndrome patients who present with near-normal counts of T-lymphocytes and their subsets. DiGeorge patients have variable clinical presentations, TREC levels and T-cell counts that are generally lower than normal, but vary considerably from patient to patient (14). However, their clinical course rarely requires HSCT. Rare cases of complete DiGeorge patients resembled SCID patients in their immunophenotype and thus pose no diagnostic challenge. While in other PID patients in our cohort, T-cell production (thymopoiesis) is not the mechanism responsible for the immunodeficiency, but their mutation leads to more complex changes broadly termed as dysregulation (WAS, ALPS, IPEX, ZAP70, CID), the SCID-RTE tube was also able to find abnormalities, mainly in the naïve/RTE compartments and in the T cell subset activation status. A CDC42 mutation in a Takenouchi-Kosaki syndrome (49, 50) patient was accidentally found in an infant with failure to thrive, lymphopenia and lymphedema by whole exome sequencing for PID suspicion. Abnormalities in lymphoid cells were clearly revealed by the PIDOT and SCID-RTE tube.

The SCID-RTE tube can also be used in patients with Combined Immunodeficiency (CID), where severe clinical presentation together with laboratory findings indicative of CID can be used for HSCT indication. ESID criteria for CID diagnosis require that apart from severe infection or immune dysregulation or affected family members, two of the four following T-cell criteria must be met: (a) reduced CD3 or CD4 or CD8 T-cells, (b) reduced naïve CD4 and/or CD8 T-cells, (c) elevated TCRγδ+T-cells, (d) reduced proliferation to mitogen or TCR stimulation (51). All three immunophenotypic criteria can be readily obtained from the SCID-RTE tube, furthermore the threshold counts of CD4+ RTE (<800 cell/μl), naïve CD4+ (<1,000 cell/μl) and naïve CD8+ lymphocytes (<290 cell/μl) are established in mutli-center and standardized diagnostic test. We would propose that the SCID-RTE tube can be used whenever there is a high clinical suspicion for SCID or CID. The SCID-RTE tube should be measured together with the PIDOT tube to obtain insight in the lymphocytes' compartment and to screen and diagnose (S)CID in a fast, standardized and efficient manner. It can also be used in a sibling of a SCID patient, immediately after birth or in children with low or absent TRECs as identified via newborn screening. SCID-RTE and PIDOT can yield the required information confirming severe T-cell abnormality or disproving it in a pre-symptomatic phase, but a separate study is needed to validate this approach in a newborn screening program. SCID-RTE can be used in patient where some abnormalities in the T-cell compartment were found by PIDOT tube. Finally, SCID-RTE and PIDOT can serve as a complementary immunophenotyping test for patients with positive TREC findings, where immunophenotyping information can serve to confirm the diagnosis of PID and direct subsequent genetic testing. Moreover, it can offer hints for the decision making process on appropriate conditioning regimen before HSCT. Importantly, the two 8-color SCRID-RTE and PIDOT tubes can also be combined into a single 12-color tube for more efficient testing.

In conclusion, we have shown that the EuroFlow SCID-RTE tube is a well-performing, fast and standardized diagnostic test for (S)CID that can be deployed in any laboratory with 8-color flow cytometer.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was approved by the local ethics committees of the participating centers [University of Salamanca, Salamanca, Spain (USAL-CSIC 20-02-2013); Charles University, Prague, Czech Republic (15-28541A); Erasmus MC, Rotterdam, The Netherlands (MEC-2013-026); University Hospital Ghent, Belgium (B670201523515) and St. Anne's University, Brno, Czech Republic (METC 1G2015)]. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR'S NOTE

All authors wish to stress that they are scientifically independent and have full freedom to act without any obligation to industry other than scientific advice to companies in the context of licensed patents. The selection of antibodies by the EuroFlow consortium is always explicitly based on quality, relevance, and continuous availability. Consequently all proposed antibody panels consist of mixtures of antibodies from many different companies.

AUTHOR CONTRIBUTIONS

MBu, TK, AO, and JD contributed to the conception and design of the study. TK, MBa, MBl, MP-A, BB, VK, CB, JP, EB, IP-K, JHMPP, BW-K, MP, JT, FH, HA, RF, TF, MS, AŠ, and SA-M performed the data acquisition and data analysis. MBa, TK, and MBu wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00371/full#supplementary-material

Supplemental Figure 1 | Detailed gating strategy.

Supplemental Figure 2 | Detailed flow cytometry dot plots for SCID patients.

Supplemental Figure 3 | Detailed flow cytometry dot plots for other PID patients.

REFERENCES

1. Fischer A, Le Deist F, Hacein-Bey-Abina S, André-Schmutz I, Basile G de Saint, de Villartay J-P, et al. Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol Rev*. (2005) 203:98–109. doi: 10.1111/j.0105-2896.2005.00223.x
2. Fischer A, Notarangelo LD, Neven B, Cavazzana M, Puck JM. Severe combined immunodeficiencies and related disorders. *Nat Rev Dis Prim*. (2015) 1:15061. doi: 10.1038/nrdp.2015.61
3. Cicalese MP, Aiuti A. Clinical applications of gene therapy for primary immunodeficiencies. *Hum Gene Ther*. (2015) 26:210–9. doi: 10.1089/hum.2015.047
4. Mamcarz E, Zhou S, Lockey T, Abdelsamed H, Cross SJ, Kang G, et al. Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. *N Engl J Med*. (2019) 380:1525–34. doi: 10.1056/NEJMoa1815408
5. Pai S-Y, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med*. (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
6. Heimall J, Logan BR, Cowan MJ, Notarangelo LD, Griffith LM, Puck JM, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. *Blood*. (2017) 130:2718–27. doi: 10.1182/blood-2017-05-781849
7. Speckmann C, Doerken S, Aiuti A, Albert MH, Al-Herz W, Allende LM, et al. A prospective study on the natural history of patients with profound combined immunodeficiency: an interim analysis. *J Allergy Clin Immunol*. (2017) 139:1302–10.e4. doi: 10.1016/j.jaci.2016.07.040
8. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet*. (2000) 355:1875–81. doi: 10.1016/S0140-6736(00)02293-5
9. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova J-L, Chatila T, et al. International union of immunological societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *J Clin Immunol*. (2018) 96–128. doi: 10.1007/s10875-017-0464-9
10. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. Phenotypic classification for primary immunodeficiencies. *J Clin Immunol*. (2018) 38:129–143. doi: 10.1007/s10875-017-0465-8
11. Yu X, Almeida JR, Darko S, Van Der Burg M, Deravin SS, Malech H, et al. Human syndromes of immunodeficiency and dysregulation are characterized by distinct defects in T-cell receptor repertoire development. *J Allergy Clin Immunol*. (2014) 133:1109–15.e14. doi: 10.1016/j.jaci.2013.11.018
12. Gennery a R. Immunological aspects of 22q11.2 deletion syndrome. *Cell Mol Life Sci*. (2012) 69:17–27. doi: 10.1007/s00018-011-0842-z
13. van der Spek J, Groenwold RHHH, van der Burg M, van Montfrans JM. TREC based newborn screening for severe combined immunodeficiency disease: a systematic review. *J Clin Immunol*. (2015) 35:416–30. doi: 10.1007/s10875-015-0152-6
14. Fronková E, Klocperk A, Svaton M, Nováková M, Kotrová M, Kayserová J, et al. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. *PLoS ONE*. (2014) 9:e114514. doi: 10.1371/journal.pone.0114514
15. 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) 132:140–50.e7. doi: 10.1016/j.jaci.2013.04.024
16. Amatuni GS, Currier RJ, Church JA, Bishop T, Grimbacher E, Nguyen AA-C, et al. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia in California, 2010-2017. *Pediatrics*. (2019) 143:2010–7. doi: 10.1542/peds.2018-2300
17. 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) 37:51–60. doi: 10.1007/s10875-016-0347-5
18. Van Der Burg M, Kalina T, Perez-Andres M, Vlkova M, Lopez-Granados E, Blanco E, et al. The EuroFlow PID orientation tube for flow cytometric diagnostic screening of primary immunodeficiencies of the lymphoid system. *Front Immunol*. (2019) 10:246. doi: 10.3389/fimmu.2019.00246
19. Müller SM, Ege M, Pottharst A, Schulz AS, Schwarz K, Friedrich W. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. *Blood*. (2001) 98:1847–51. doi: 10.1182/blood.V98.6.1847
20. Wahlstrom J, Patel K, Eckhert E, Kong D, Horn B, Cowan MJ, et al. Transplacental maternal engraftment and posttransplantation graft-versus-host disease in children with severe combined immunodeficiency. *J Allergy Clin Immunol*. (2017) 139:628–33.e10. doi: 10.1016/j.jaci.2016.04.049
21. Roifman CM, Somech R, Kavadas F, Pires L, Nahum A, Dalal I, et al. Defining combined immunodeficiency. *J Allergy Clin Immunol*. (2012) 130:177–83. doi: 10.1016/j.jaci.2012.04.029
22. Appay V, van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A*. (2008) 73:975–83. doi: 10.1002/cyto.a.20643
23. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol*. (2013) 43:2797–809. doi: 10.1002/eji.201343751
24. Livak F, Schatz DG. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. *Mol Cell Biol*. (1996) 16:609–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8552089 doi: 10.1128/MCB.16.2.609
25. Kimmig S, Przybylski GK, Schmidt C A, Laurisch K, Möwes B, Radbruch A, et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med*. (2002) 195:789–94. doi: 10.1084/jem.20011756
26. Kohler S, Thiel A. Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. *Blood*. (2009) 113:769–74. doi: 10.1182/blood-2008-02-139154
27. de Saint-Basile G, Le Deist F, de Villartay JP, Cerf-Bensussan N, Journet O, Brousse N, et al. Restricted heterogeneity of T lymphocytes in combined immunodeficiency with hypereosinophilia. (Omenn's syndrome). *J Clin Invest*. (1991) 87:1352–9. doi: 10.1172/JCI115139
28. van der Velden VHJ, Flores-Montero J, Perez-Andres M, Martin-Ayuso M, Crespo O, Blanco E, et al. Optimization and testing of dried antibody tube: the EuroFlow LST and PIDOT tubes as examples. *J Immunol Methods*. (2019) 475:112287. doi: 10.1016/j.jim.2017.03.011
29. Kalina T, Flores-Montero J, van der Velden VHJ, Martin-Ayuso M, Böttcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia*. (2012) 26:1986–2010. doi: 10.1038/leu.2012.122
30. Flores-Montero J, Sanoja-Flores L, Paiva B, Puig N, García-Sánchez O, Böttcher S, et al. Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia*. (2017) 31:2094–103. doi: 10.1038/leu.2017.29
31. Glier H, Novakova M, te Marvelde J, Bijkerk A, Morf D, Thurner D, et al. Comments on EuroFlow standard operating procedures for instrument setup and compensation for BD FACS Canto II, Navios and BD FACS lyric instruments. *J Immunol Methods*. (2019) 475:112680. doi: 10.1016/j.jim.2019.112680

32. Magerus-Chatinet A, Stolzenberg M-C, Loffredo MS, Neven B, Schaffner C, Ducrot N, et al. FAS-L, IL-10, and double-negative CD4–CD8– TCR α/β+ T cells are reliable markers of autoimmune lymphoproliferative syndrome. (ALPS) associated with FAS loss of function. *Blood*. (2009) 113:3027–30. doi: 10.1182/blood-2008-09-179630
33. Morio T, Takase K, Okawa H, Oguchi M, Kanbara M, Hiruma F, et al. The increase of non-MHC-restricted cytotoxic cells. (gamma/delta-TCR-bearing T cells or NK cells) and the abnormal differentiation of B cells in Wiskott-Aldrich syndrome. *Clin Immunol Immunopathol*. (1989) 52:279–90. doi: 10.1016/0090-1229(89)90179-7
34. van Gent R, van Tilburg CM, Nibbelke EE, Otto SA, Gaiser JF, Janssens-Korpela PL, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. *Clin Immunol*. (2009) 133:95–107. doi: 10.1016/j.clim.2009.05.020
35. Schatorjé EJH, Gemen EFA, Driessen GJA, Leuvenink J, van Hout RWNM, de Vries E. Paediatric reference values for the peripheral T cell compartment. *Scand J Immunol*. (2012) 75:436–44. doi: 10.1111/j.1365-3083.2012.02671.x
36. Comans-Bitter WM, De Groot R, Van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood: reference values for lymphocyte subpopulations. *J Pediatr*. (1997) 130:388–93. doi: 10.1016/S0022-3476(97)70200-2
37. Takashima T, Okamura M, Yeh T-W, Okano T, Yamashita M, Tanaka K, et al. Multicolor flow cytometry for the diagnosis of primary immunodeficiency diseases. *J Clin Immunol*. (2017) 37:486–95. doi: 10.1007/s10875-017-0405-7
38. Van Dongen JJMM, Van Der Burg M, Kalina T, Perez-Andres M, Mejstrikova E, Vlkova M, et al. EuroFlow-based flowcytometric diagnostic screening and classification of primary immunodeficiencies of the lymphoid system. *Front Immunol*. (2019) 10:1271. doi: 10.3389/fimmu.2019.01271
39. Blanco E, Pérez-Andrés M, Arriba-Méndez S, Contreras-Sanfeliciano T, Criado I, Pelak O, et al. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. *J Allergy Clin Immunol*. (2018) 141:2208–19.e16. doi: 10.1016/j.jaci.2018.02.017
40. Villa A, Notarangelo LD, Roifman CM. Omenn syndrome: inflammation in leaky severe combined immunodeficiency. *J Allergy Clin Immunol*. (2008) 122:1082–6. doi: 10.1016/j.jaci.2008.09.037
41. IJspeert H, Driessen GJ, Moorhouse MJ, Hartwig NG, Wolska-Kusnierz B, Kalwak K, et al. Similar recombination-activating gene. (RAG) mutations result in similar immunobiological effects but in different clinical phenotypes. *J Allergy Clin Immunol*. (2014) 133:1124–33. doi: 10.1016/j.jaci.2013.11.028
42. Hazenberg MD, Borghans J a M, de Boer RJ, Miedema F. Thymic output: a bad TREC record. *Nat Immunol*. (2003) 4:97–9. doi: 10.1038/ni0203-97
43. Tanaskovic S, Fernandez S, Price P, Lee S, French MA. CD31. (PECAM-1) is a marker of recent thymic emigrants among CD4+ T-cells, but not CD8+ T-cells or gammadelta T-cells. *Immunol Cell Biol*. (2010) 88:321–7. doi: 10.1038/icb.2009.108
44. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med*. (2001) 79:631–40. doi: 10.1007/s001090100271
45. Hazenberg MD, Otto S a, de Pauw ES, Roelofs H, Fibbe WE, Hamann D, et al. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. *Blood*. (2002) 99:3449–53. doi: 10.1182/blood.V99.9.3449
46. Hazenberg MD, Otto SA, Cohen Stuart JW, Verschuren MC, Borleffs JC, Boucher C a, et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med*. (2000) 6:1036–42. doi: 10.1038/79549
47. Junge S, Kloeckener-Gruissem B, Zufferey R, Keisker A, Salgo B, Fauchere J-C, et al. Correlation between recent thymic emigrants and CD31+ (PECAM-1) CD4+ T cells in normal individuals during aging and in lymphopenic children. *Eur J Immunol*. (2007) 37:3270–80. doi: 10.1002/eji.200636976
48. Game DS, Rogers NJ, Lechler RI. Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells. *Am J Transplant*. (2005) 5:1614–25. doi: 10.1111/j.1600-6143.2005.00916.x
49. Martinelli S, Krumbach OHF, Pantaleoni F, Coppola S, Amin E, Pannone L, et al. Functional dysregulation of CDC42 causes diverse developmental phenotypes. *Am J Hum Genet*. (2018) 102:309–20. doi: 10.1016/j.ajhg.2017.12.015
50. Takenouchi T, Kosaki R, Niizuma T, Hata K, Kosaki K. Macrothrombocytopenia and developmental delay with a *de novo* CDC42 mutation: yet another locus for thrombocytopenia and developmental delay. *Am J Med Genet A*. (2015) 167A:2822–5. doi: 10.1002/ajmg.a.37275
51. Seidel MG, Kindle G, Gathmann B, Quinti I, Buckland M, van Montfrans J, et al. The European Society for Immunodeficiencies. (ESID) registry working definitions for the clinical diagnosis of inborn errors of immunity. *J allergy Clin Immunol Pract*. (2019) 7:1763–70. doi: 10.1016/j.jaip.2019.02.004

Conflict of Interest: JD, AO, MBu, MP-A, TK, and EB each report being one of the inventors on the EuroFlow-owned patent PCT/NL 2015/ 050762 (Diagnosis of primary immunodeficiencies). The Infinicyt software is based on intellectual property (IP) of some EuroFlow laboratories (University of Salamanca in Spain and Federal University of Rio de Janeiro in Brazil) and the scientific input of other EuroFlow members. All above mentioned intellectual property and related patents are licensed to Cytognos (Salamanca, ES), which pays royalties to the EuroFlow Consortium. These royalties are exclusively used for continuation of the EuroFlow collaboration and sustainability of the EuroFlow consortium. JD and AO report an Educational Services Agreement from BD Biosciences (San José, CA) and a Scientific Advisor Agreement with Cytognos; all related fees and honoraria are for the involved university departments at Leiden University Medical Center and University of Salamanca, respectively.

The remaining 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.

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Information and Emotional Support Needs of Families Whose Infant Was Diagnosed With SCID Through Newborn Screening

Melissa Raspa[1], Molly Lynch[1], Linda Squiers[1], Angela Gwaltney[1], Katherine Porter[1], Holly Peay[1], Alissa Huston[2], Brian Fitzek[2] and John G. Boyle[2]*

[1] RTI International Research Triangle Park, Durham, NC, United States, [2] Immune Deficiency Foundation Towson, Towson, MD, United States

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Edited by:
Antonio Condino-Neto, University of São Paulo, Brazil

Reviewed by:
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Surjit Singh, Post Graduate Institute of Medical Education and Research (PGIMER), India

****Correspondence:***
Melissa Raspa
mraspa@rti.org

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Background: Now that severe combined immune deficiency (SCID) has been added to newborn screening panels in all 50 states in the U.S., there is a need to develop and disseminate well-designed educational materials to parents who need information to make informed decisions about treatment and care for identified infants. SCID Compass was designed to address this gap. We summarize the results of two needs assessment activities for parents—a journey mapping exercise and online survey—which will inform the development of a website and new resources.

Methods: We conducted in-depth interviews with seven parents of children with SCID identified through newborn screening. Participants were asked to complete a journey map to describe key timepoints related to SCID, starting at diagnosis through present day. This qualitative information informed an online survey that was completed by 76 parents who had a child with SCID. All participants were from the United States.

Results: Analysis of journey maps revealed five distinct stages that parents experience: (1) Diagnosis, (2) Pre-Treatment, (3) Treatment, (4) Post-Treatment, and (5) The New Normal. At each stage, parents described unique emotions, challenges, contextual factors that can make a difference in their experience, and information and resource needs. Survey results indicated the highest-rated information needs for parents were understanding available treatment options and what to expect across the SCID lifespan. Emotional support needs included dealing with uncertainty about child's future and additional opportunities to connect with other families. Parents preferred receiving new materials from their healthcare provider or other families, and preferred materials in print, from social media, or online. Several differences were found among subgroups of parents, including those whose child had been identified through newborn screening as well as those considered medically underserved.

Conclusions: Findings about unmet parent needs and informational preferences will serve as the foundation for creating a suite of resources for those who have a child

with SCID. The materials will be tailored to specific stages of the journey. By using a family-centered approach, we will help to ensure that the materials designed and developed as part of SCID Compass will be understandable, comprehensive, and useful.

Keywords: severe combined immunodeficiency, newborn screening, parents and families, information needs, emotional needs, medically underserved, educational materials

INTRODUCTION

Severe combined immune deficiency (SCID) is a group of genetic disorders that cause profound defects in cellular and humoral immunity characterized by a deficiency or absence of T-cells (1, 2). Patients with SCID are highly susceptible to severe and recurrent infections, which can result in significant morbidity or mortality in infancy. SCID is considered a rare disease, with prevalence estimates of ∼ 1 in 58,000 births (3, 4).

Historically, early clinical diagnosis of SCID was delayed due to a lack of family history or absence of distinguishing symptoms given that infections are common in pediatric populations. However, several studies demonstrated that early identification and provision of immune reconstituting treatment, such as bone marrow transplantation, are highly effective in reducing illness and death in patients with SCID (3, 5–7). Thus, after a robust evidence review by the Advisory Committee on Heritable Disorders in Newborns and Children (8), the United States Secretary of Health and Human Services recommended that all states add SCID and related T-cell lymphocyte deficiencies to their uniform newborn screening panel. As of December 2018, all states have fully implemented newborn screening for SCID. As a result, more children are benefiting from earlier, pre-symptomatic treatment (9).

Despite moving forward with earlier identification, the SCID community continues to face a number of significant challenges, such as a lack of communication between the newborn screening labs, healthcare providers, and families; disparities in knowledge and care for patients with SCID in rural and underserved communities; and generally low awareness and knowledge about SCID and SCID newborn screening for all stakeholders (10). In July 2018, the Health Services and Resources Administration (HRSA) funded a project to directly address the ongoing needs of the SCID community. The goal of the program is to improve the outcomes of infants with SCID detected through newborn screening by increasing awareness and knowledge about SCID; supporting state newborn screening programs; linking families, especially those living in rural and medically underserved areas, to services; and developing long-term follow-up strategies for infants identified through newborn screening. One of the cornerstones of the project, branded SCID Compass, will be a new web site that will house educational materials for parents and all stakeholders.

As part of the family-centered web site development process, the project team wanted to gain a better understanding of unmet educational and support needs and information preferences for parents. However, few studies exist on the information or emotional support needs of parents of children with SCID. A qualitative study of 11 parents of children with SCID reported some common psychosocial stressors and challenges, including the feelings of loss of normalcy and lack of control over multiple aspects of life, the ongoing stress of waiting (both between diagnosis and treatment and between treatment and evidence of treatment efficacy), and the lack of information on how to cope with isolation while in the hospital and after the transition to home (11). Another study suggested that parents of children with SCID who were diagnosed through newborn screening may have higher levels of postpartum depression and posttraumatic stress disorder (12). These findings echo those of a survey of parents who have a child with a rare disease which reported high rates of financial challenges, difficulty in accessing disease-specific support groups, and feelings of isolation, anxiety, and uncertainty (13).

Given the lack of guidance found in the literature, a critical analysis of the challenges faced by parents of a child with SCID was needed. By analyzing the experiences and needs of parents of a child with SCID, we will be able to develop much-needed education materials and ultimately help healthcare providers and the broader newborn screening community to provide more appropriate support and resources. The following questions were addressed as part of the needs assessment:

1. What are the experiences of a parents who has a child with SCID, from diagnosis through post-treatment?
2. What are the informational and emotional support needs of parents of a child with SCID and what are the desired points in time to receive this information and support?
3. Are there any differences in informational and emotional support needs by subgroups of families, specifically those whose child was diagnosed through newborn screening or those who are medically underserved?
4. How do parents rate the available SCID-related materials and what are their preferred sources and formats of materials?

MATERIALS AND METHODS

Design

To answer these questions, we used a two-step, mixed-method approach. First, we conducted a journey mapping activity with a small group of parents who had a child with SCID. A journey map is a diagram or visual representation of a person's experience as they go through a process, often depicted in stages or key points in time (14). As a tool within a user-centered design methodology, journey mapping provides members of the target audience the opportunity to describe their experience from beginning to end, including important milestones, challenges, and successes. Using the qualitative information from the journey mapping activity, we next developed a complementary

quantitative survey to expand upon the journey map findings with a broader sample of participants. The goal of both the journey map and the survey was to gain a better understanding of parent's information and emotional support needs and identify opportunities for interventions at key points in time along the journey.

Participants

Parents were recruited in partnership with the two patient advocacy organizations. The Immune Deficiency Foundation (IDF) and SCID Angels for Life Foundation contacted parents through their Facebook groups, email listservs, and personal calls. Parents who were interested in participating in the journey mapping were referred to study staff to schedule interviews. To be eligible, parents had to be 18 years of age, speak English, and have had a child with SCID identified through newborn screening. A total of seven parents participated in the journey mapping activity.

For the online survey, parents were contacted through similar methods and then directed to the web site to participate. Eligibility criteria for the survey included being at least 18 years of age and having a child with SCID; children did not have to be identified through newborn screening. A total of 76 parents met the eligibility criteria and completed the survey. **Table 1** provides an overview of the participants. All participants were from the United States.

Instruments and Procedures

Prior to the journey mapping interview, we emailed participants a worksheet with six empty boxes that fit together to look like a pathway. Using written instructions, we asked participants to think back to the point in time when they first learned their child screened positive for SCID and to select the six points in time when they experienced the greatest successes or challenges related to their child's diagnosis, treatment, and ongoing management of the condition. We also asked about their key information needs and resources they used to meet these needs, and what else would have been helpful. Next, we conducted in-depth interviews using the journey map worksheets to guide the discussion. We asked participants to describe the six time points they selected and share their reasons for including them on their journey map. Approximately 2 weeks after the interviews were completed, we convened all participants in a focus group to review and validate a group-level journey map that we developed based on a synthesis of individual responses. A trained moderator used a semi-structured guide during the interviews and focus groups while a staff member took detailed notes. Participants were offered an incentive to participate in the journey mapping activity.

Approximately 3 months after the journey mapping activities, we launched the online survey. The survey was programmed into Survey Gizmo and was available online in English and Spanish. For those who did not have access to computers or the Internet (e.g., Amish or Mennonite families), a paper version was also available. Printed copies of the survey were mailed to interested participants with a postage paid return envelope. Completed surveys were then entered into Survey Gizmo on behalf of the

TABLE 1 | Description of survey participants and their child with SCID.

Child and family characteristics	*N*	%
Relationship to SCID child		
→ Father	10	13.16%
→ Mother	66	86.84%
Race		
→ White	62	81.58%
→ Black or African American	2	2.63%
→ Asian	1	1.32%
→ Other race	3	3.95%
→ More than one race	6	7.89%
→ Prefer not to say	2	2.63%
Ethnicity		
→ Hispanic or Latino	8	10.53%
→ Not Hispanic or Latino	67	88.16%
→ Prefer not to say	1	1.32%
Live in "Immunology Desert"[a]		
→ No	64	84.21%
→ Yes	12	15.79%
Area description		
→ Urban (large or small city)	34	44.74%
→ Suburban (town outside city limits)	26	34.21%
→ Rural (small community)	16	21.05%
Highest level of education completed		
→ 8th grade or less	7	9.21%
→ Some HS but did not graduate	2	2.63%
→ HS or GED	7	9.21%
→ Some college or 2-year degree	20	26.32%
→ 4-year college graduate	19	25.00%
→ More than 4-year college degree	21	27.63%
Children		
→ Total number of children (Mean, SD)	2.62	1.92
→ Total children with SCID (Mean, SD)	1.28	0.78
→ Children who died from SCID (Mean, SD)	0.20	0.52
→ Age of Child (at time of survey completion)	10.92	10.59
Child's SCID type		
→ Common Gamma Chain of the T-Cell Receptor (XSCID/IL2RG)	32	42.11%
→ Deficiency of the Alpha Chain of the IL-7 Receptor (IL-7Rα)	5	6.58%
→ Deficiency of Janus Kinase 3 (JAK3)	2	2.63%
→ Deficiency of the CD3 Chains	1	1.32%
→ RAG1/RAG2	11	14.47%
→ ADA Deficiency	12	15.79%
→ DCLRE1C (Artemis) Mutation	2	2.63%
→ Other	2	2.63%
→ Unknown	9	11.84%
Child diagnosed with Omenn Syndrome		
→ Yes	2	2.63%
→ No	69	90.79%
→ I don't know	5	6.58%
Diagnosed through newborn screening		
→ Yes	34	44.74%
→ No	42	55.26%

[a] *Immunology Desert is defined as having a home zip code more than 150 miles from a primary immunodeficiency clinic.*

participant. Data collection was open for about 3 months. The first section of the survey asked questions to gather child and family demographic information. Next, we asked participants to report on their information and emotional support needs. A series of needs were listed; participants were asked whether each was ever a large need, a small need, or not a need. We also asked participants to indicate where they turn to for information about SCID and rate the quality of SCID materials they most recently accessed. Finally, we asked participants about their preferred format and source of materials.

Data Analysis

The data obtained from the journey mapping interviews were organized using a meta-matrix (15). We then conducted a thematic analysis to identify common stages of a participants' journeys and distill key findings, such as emotional and practical challenges, information needs, and facilitators at each point in time (16). Upon analyzing the seven interviews, we determined that saturation was reached given that participants described similar phases of their journey despite their unique experiences (17). Using findings from the thematic analysis, we created a group-level journey map. We presented the compiled journey map to the interview participants in a virtual group discussion as a validity check.

Survey data were analyzed using SAS Grid Manger, version 7.15 (2017, Cary, NC). Descriptive data include frequencies, means, and standard deviations. In order to calculate mean scores for the informational and emotional support needs items, we scored each need as follows: 0 = Not a need, 1 = A small need, 2 = A large need. For the mean scores that rated the quality of information, we used the following scale: (a) Usefulness: 1 = Not at all useful to 7 = Extremely useful, (b) Ability to understand: 1 = Extremely difficult to 7 = Extremely easy, (c) Ability to find: 1 = Extremely difficult to 7 = Extremely easy, and (d) Trustworthiness: 1 = Very untrustworthy to 7 = Very trustworthy. Parent's rating of their preference for source and format of material was coded as 1 = Not at all interested to 7 = Extremely interested.

In order to examine subgroup group differences, we split parents into dichotomous groups. The first examined differences in needs between those who had a child identified through newborn screening and those whose children were diagnosed clinically. We also wanted to understand any differences in needs between parents who may be medically underserved and those who were not. Thus, we created three different groupings of parents using the race/ethnicity, education level, and geographic location variables as indicators of being medically underserved. Specifically, we defined the groups as (a) non-White or Hispanic parents vs. White, non-Hispanic, (b) those with less than a 4-year college degree vs. those with a bachelors or graduate degree, and (c) those living in a rural area or immunology desert (home zip code more than 150 miles from a primary immunodeficiency clinic) vs. those who were living in an urban or suburban setting and were not in an immunology desert. For each subgroup, parents who met the first criteria (e.g., non-White, Hispanic) were considered medically underserved. *T*-tests were used to detect group differences between mean scores for each of the informational and emotional support needs. Pooled (equal) or Satterthwaite (unequal) test statistics are reported depending on the equality of variances test.

RESULTS

Journey Map Findings

The goal of the journey mapping activity was to better understand the key experiences of parents of a child with SCID, from initial diagnosis through present day. Analysis revealed five stages that all parents experienced after receiving a positive newborn screen for SCID, as depicted in **Figure 1**. Each stage is described below, with descriptions of challenges and specific needs at each point in time.

Stage 1: Diagnosis. In this first stage, the infant receives a positive screen and diagnosis is later confirmed. Parents described how their world is turned upside down as they undergo a transition from seeing their newborn as healthy to attempting to understand a surprising diagnosis. The main challenges stem from a lack of readily available, comprehensive resources about SCID and unclear communication from their child's pediatrician or other healthcare providers. However, the provision of clear and useful information from their pediatrician and a smooth referral process to a specialist helped to ameliorate some of these challenges. At this stage, the primary information needs were up-to-date resources about SCID, connections to other families, as well as success stories demonstrating that SCID is treatable.

Stage 2: Pre-treatment. After referral to a specialist, infants are often admitted to the hospital and placed in isolation; they may also be isolated at home. During this time, the healthcare team selects and prepares for a treatment, such as a bone marrow transplant or gene therapy. For bone marrow transplants, typically there is a waiting period while a donor is identified. Parents described challenges around healthcare providers' lack of knowledge of SCID, particularly when nurses or technicians in the hospital do not comply with isolation and contact protocols. Parents also described frustration with the lack of national standards for SCID treatment, noting that treatment decisions are specific to the hospital. Primary information needs in this stage revolved around the benefits and risks of all possible treatments options and getting connected to other families who have gone through the process.

> *"Throughout the journey, the bone marrow transplant makes you feel so helpless. If there is no donor what do you do? You just keep waiting. I feel like a lot of education work needs to be done in that area."*

Stage 3: Treatment. The third stage focuses on the treatment procedure, including conditioning (e.g., chemotherapy). Parents described continuing challenges around the lack of information about treatments for SCID, particularly what to expect during and after treatment. For example, several participants mentioned that there are resources about bone marrow transplants, but almost none of this information is applicable to infants. Available resources often describe transplants for older children or adolescents with leukemia. Parents also described the strain on

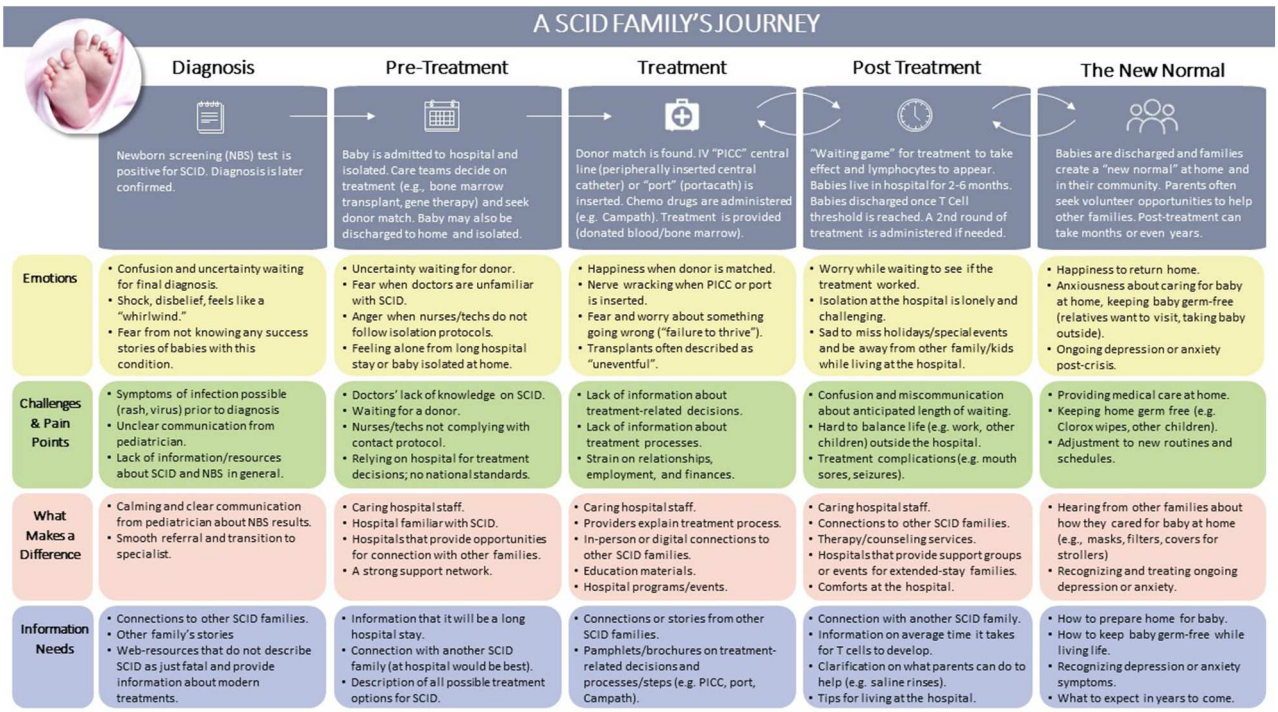

FIGURE 1 | Results of journey map activity.

relationships, employment, and finances as the journey continues several months or more. Parents underscored the need for up-to-date and detailed information about treatment options, processes, and recovery in this stage.

> *"His type of SCID is very rare so we didn't know what was going to be the right course of action for him. There was nothing helpful to me. Maybe if parents could be provided brochures with different families' testimonies and stories. I think that would be very helpful for parents going through it now. I didn't know there were so many varieties of SCID and so many treatments for each category. Some can get the newer ones now. I didn't have any of that information."*

Stage 4: Post Treatment. After their infant received treatment, parents anxiously waited to see if the treatment would be effective. This period can take up to several months, and typically the infant is hospitalized during this process. Parents described the high levels of worry and loneliness during this period, sadness to miss holidays and family events, and frustration at the long duration of the hospital stay. Information needs centered around the anticipated length of time to see if lymphocytes appear, guidance and tips for living in the hospital, and continuing connections with other families who have gone through this process.

> *"They were doing bloodwork almost every week. You're waiting for changes but there weren't changes; the levels were still the same. It was hard. It was just a waiting game. Waiting to see when I would be out of the hospital or how long I would be there."*

Stage 5: The New Normal/Living with SCID. Parents described this stage as the transition back to home and community and living the "new normal." Parents described a mix of emotions—relief and joy about leaving the hospital and surviving isolation, but anxiety about caring for their child at home and maintaining strict germ-free protocols around their other children, relatives, and during day-to-day life. At this stage, several parents recognized symptoms of depression or anxiety that emerged in this post-crisis period. Others described a need to "give back" in this stage and sought opportunities to volunteer or connect with families beginning their journey. Information needs centered around how to prepare their home for their child after treatment, what to do about ongoing depression and anxiety, and what to expect in years to come.

> *"We were officially off isolation and took him to dinner, but I was so nervous to take him out. I wiped the entire table with Lysol and put a mask on him."*

Survey Findings

Shortly after the completion of the journey mapping activity, we launched the survey to assess a broad range of informational and emotional support needs. We also asked parents to tell us about their experiences in seeking SCID-related information and their preferred source and format of materials.

Informational support. **Table 2** presents means ratings by parents for informational support needs. For all parents, the highest rated informational support needs were: (a)

TABLE 2 | Total mean (SD) parent ratings for informational and emotional support needs and by comparison groups.

	All parents	Newborn screening		Education level		Rural or immunology desert	
	Total ($n = 76$)	Yes ($n = 34$)	No ($n = 42$)	Less ($n = 36$)	More ($n = 40$)	Yes ($n = 5$)	No ($n = 71$)
Informational Needs							
Understanding newborn screening results	1.49 (0.81)	1.74 (0.57)	1.29 (0.92)*	1.42 (0.84)	1.55 (0.78)	1.60 (0.89)	1.48 (0.81)
Understanding specific type of child's SCID	1.75 (0.59)	1.79 (0.54)	1.71 (0.64)	1.69 (0.67)	1.80 (0.52)	1.80 (0.45)	1.75 (0.60)
Understanding what to expect across SCID lifespan	1.89 (0.39)	1.94 (0.34)	1.86 (0.42)	1.83 (0.51)	1.95 (0.22)	2.00 (0.00)	1.89 (0.40)*
Understanding all available treatment options	1.91 (0.37)	1.97 (0.17)	1.86 (0.47)	1.89 (0.40)	1.93 (0.35)	2.00 (0.00)	1.90 (0.38)*
Knowing where to access specialists	1.79 (0.50)	1.76 (0.50)	1.81 (0.51)	1.78 (0.59)	1.80 (0.41)	2.00 (0.00)	1.77 (0.51)***
Knowing what to expect during treatment and hospital stay	1.87 (0.44)	1.91 (0.29)	1.83 (0.54)	1.92 (0.37)	1.83 (0.50)	2.00 (0.00)	1.86 (0.46)*
Understanding managing treatment side-effects	1.72 (0.53)	1.74 (0.51)	1.71 (0.55)	1.81 (0.52)	1.65 (0.53)	2.00 (0.00)	1.70 (0.54)***
Knowing how to keep child healthy after treatment	1.83 (0.41)	1.82 (0.39)	1.83 (0.44)	1.81 (0.47)	1.85 (0.36)	1.80 (0.45)	1.83 (0.41)
Knowing where to seek financial assistance	1.58 (0.62)	1.47 (0.61)	1.67 (0.61)	1.69 (0.62)	1.48 (0.60)	1.60 (0.89)	1.58 (0.60)
Knowing how family can adapt	1.64 (0.53)	1.68 (0.47)	1.62 (0.58)	1.61 (0.60)	1.68 (0.47)	1.80 (0.45)	1.63 (0.54)
Understanding the chance of having another child with SCID	1.45 (0.76)	1.56 (0.75)	1.36 (0.76)	1.72 (0.61)	1.20 (0.79)**	1.40 (0.89)	1.45 (0.75)
Understanding alternative methods of having another child	1.05 (0.85)	1.26 (0.79)	0.88 (0.86)*	1.08 (0.87)	1.03 (0.83)	1.40 (0.89)	1.03 (0.84)
Knowing how to educate family and friends about SCID	1.58 (0.85)	1.53 (0.61)	1.62 (0.62)	1.67 (0.63)	1.50 (0.60)	1.80 (0.45)	1.56 (0.63)
Knowing how to talk to my other children about SCID	0.96 (0.86)	0.97 (0.87)	0.95 (0.85)	1.25 (0.81)	0.70 (0.82)**	1.00 (1.00)	0.96 (0.85)
Knowing how to educate hospital staff about SCID	1.49 (0.66)	1.35 (0.65)	1.60 (0.66)	1.47 (0.77)	1.50 (0.55)	1.60 (0.55)	1.48 (0.67)
Knowing how to educate my child's PCP about SCID	1.47 (0.70)	1.38 (0.70)	1.55 (0.71)	1.56 (0.73)	1.40 (0.67)	1.80 (0.45)	1.45 (0.71)
Emotional support needs							
Opportunity to talk to other families	1.76 (0.49)	1.79 (0.48)	1.74 (0.50)	1.75 (0.50)	1.78 (0.48)	2.00 (0.00)	1.75 (0.50)***
Dealing with uncertainty about child's future	1.79 (0.50)	1.94 (0.24)	1.67 (0.61)*	1.69 (0.58)	1.88 (0.40)	2.00 (0.00)	1.77 (0.51)***
Managing my emotions as parent/caregiver	1.70 (0.65)	1.85 (0.44)	1.57 (0.77)*	1.72 (0.66)	1.68 (0.66)	2.00 (0.00)	1.68 (0.67)***
Understanding importance of self-care	1.59 (0.61)	1.68 (0.53)	1.52 (0.67)	1.58 (0.65)	1.60 (0.59)	2.00 (0.00)	1.56 (0.63)***
Getting support needed from family or friends	1.57 (0.62)	1.56 (0.56)	1.57 (0.67)	1.69 (0.58)	1.45 (0.64)	1.80 (0.45)	1.55 (0.63)
Managing changes in relationship with partner	1.37 (0.78)	1.44 (0.70)	1.31 (0.84)	1.39 (0.80)	1.35 (0.77)	2.00 (0.00)	1.32 (0.79)***
Managing changes in relationship with other children	0.85 (0.91)	0.91 (0.93)	0.80 (0.90)	1.17 (0.92)	0.58 (0.81)**	1.20 (1.10)	0.83 (0.90)
Managing changes in relationship with extended family	1.05 (0.79)	1.03 (0.80)	1.07 (0.79)	1.08 (0.84)	1.03 (0.74)	1.60 (0.55)	1.01 (0.79)
Having access to professional counseling	1.32 (0.77)	1.38 (0.70)	1.26 (0.83)	1.22 (0.87)	1.40 (0.67)	1.80 (0.45)	1.28 (0.78)
Having access to professional organizations knowledgeable about SCID	1.74 (0.53)	1.76 (0.50)	1.71 (0.55)	1.61 (0.64)	1.85 (0.36)	2.00 (0.00)	1.72 (0.54)***

0, Not a need; 1, A small need; 2, A large need.
**$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Understanding all available treatment options (1.91), (b) Understanding what to expect across the SCID lifespan (1.89), (c) Knowing what to expect during treatment and hospital stay (1.87), (d) Knowing how to keep child healthy after treatment (1.83), and (e) Knowing where to access specialists (1.79). The lowest-rated information needs were (a) Knowing how to talk to my other children about SCID (0.96), Understanding alternative methods of having another child (1.05), and (c) Understanding the chance of having another child with SCID (1.45).

When we explored subgroups of parents, we found several noteworthy findings that indicated different informational support needs for those who had a child diagnosed through newborn screening or those who were medically underserved (see **Table 2**). For parents who had a child diagnosed through newborn screening, there were two statistically significant findings showing higher informational needs than parents whose child was diagnosed clinically: (a) Understanding newborn screening results [$t_{[69.559]} = 2.62$, $p = 0.0109$] and (b) Understanding alternative methods of having another child (e.g., *in-vitro* fertilization) [$t_{[74]} = 2.02$, $p = 0.0488$]. When examining differences by education level, parents with less education had more informational needs for (a) Understanding the chance of having another child with SCID [$t_{[74]} = 3.19$, $p = 0.00021$] and (b) Knowing how to talk to their other children about SCID [$t_{[74]} = 2.94$, $p = 0.0044$]. Several statistically significant differences were found between those parents who were living in a rural area or immunology desert when compared with those who were not. Specifically, those in a rural area or an immunology desert had more informational support needs for: (a) Understanding what to expect across SCID lifespan [$t_{[70]} = -2.38$, $p = 0.0198$], (b) Understanding all available treatment options [$t_{[70]} = -2.16$, $p = 0.0338$], (c) Knowing where to access specialists [$t_{[70]} = -3.70$, $p = 0.0004$], (d) Knowing what to expect during treatment and hospital stay [$t_{[70]} = -2.60$, $p = 0.0114$], and (e) Understanding

how to manage treatment side-effects [$t_{[70]} = -4.57$, $p < 0.0001$]. There were no differences in informational support needs when comparing parents who were non-White, Hispanic with those who were not.

Emotional support. As shown in **Table 2**, the highest rated emotional support needs for all parents were: (1) Dealing with uncertainty about child's future (1.79), (b) Having the opportunity to talk to other families (1.76), (c) Having access to professional organization knowledgeable about SCID (1.74), (d) Managing my emotions as parent/caregiver (1.70), and (e) Understanding the importance of self-care (1.59). The lowest rated items were (a) Managing changes in relationships with other children (0.85), (b) Managing changes in relationship with extended family (1.05), and (c) Managing changes in relationship with partner (1.37).

Emotional support needs also differed by several of the parent subgroups (see **Table 2**). For those who had a child diagnosed through newborn screening, the following emotional supports needs were rated higher than those who did not have a child diagnosed through newborn screening: (a) Dealing with uncertainty about child's future [$t_{[55.459]} = 2.67$, $p < 0.0001$], and (b) Managing my emotions as a parent/caregiver [$t_{[66.869]} = 2.01$, $p = 0.0489$]. Parents who had less education reported that managing changes in relationship with other children was more of a need than those with more education [$t_{[73]} = 2.98$, $p = 0.0040$]. Several emotional support needs were higher for those who were living in a rural area or an immunology desert, including (a) Having the opportunity to talk to other families [$t_{[70]} = -4.28$, $p < 0.0001$], (b) Dealing with uncertainty about child's future [$t_{[70]} = -3.70$, $p = 0.0004$], (c) Managing my emotions as parent/caregiver [$t_{[70]} = -4.07$, $p < 0.0001$], (d) Understanding the importance of self-care [$t_{[70]} = -5.87$, $p < 0.0001$], (e) Managing changes in relationship with partner [$t_{[70]} = -7.22$, $p < 0.0001$], and (f) Having access to professional organizations knowledgeable about SCID [$t_{[70]} = -4.40$, $p < 0.0001$]. Similar to the informational support needs, there were no group differences in emotional support needs by parents' race or ethnicity.

Information seeking. Parents reported on their most recent experience with seeking information about SCID. Almost a third of participants (30%) reported searching for information within the last 30 days. Another 22% searched for information in the past 1–3 months; 12% in the past 4–6 months, 17% more than 6 months ago, and 18% said they couldn't remember. When asked what topics they were searching for, many parents stated they wanted more information on treatment options, complications related to treatment, or long-term outcomes related to treatments. Others mentioned searching for the latest research while others wanted to connect to other families or were looking for other types of informational or emotional support. Almost half (45%) of parents mentioned that they found the information they were looking for. Over a quarter of parents (28%), though, stated that their search for the specific SCID information they wanted was not successful. The remainder (25%) couldn't remember if they had found the information.

When asked about the quality of the information they had found ($n = 34$), parents rated them as very trustworthy (mean = 6.18, $SD = 1.24$). Slightly lower scores were reported for the usefulness (mean = 5.61, $SD = 1.25$) and ability to understand (mean = 5.55, $SD = 1.23$) the information. The lowest rated dimension was the ability to find the information parents were looking for (mean = 5.15, $SD = 1.62$). White, non-Hispanics were slightly less likely to consider the material that they found as trustworthy than non-White/Hispanics [$t_{[30.694]} = 2.37$, $p = 0.0242$]. There were no subgroup differences between parents who had a child diagnosed through newborn screening and those who did not, between parents with lower or higher education levels, or between parents who lived in a rural area or immunology dessert and those who did not.

Preferred format and source of materials. **Table 3** provides information about parents' preferred formats and sources of materials about SCID. Parents rated receiving information in-person from either healthcare providers or other parents the highest. Reading printed information, interacting on social media, and reading a web site were the next preferred formats. The lowest rated formats were videos and podcasts. Non-whites, Hispanics were more likely to prefer to obtain their information from professionals in person [$t_{[39.373]} = 2.18$, $p < 0.0350$]. Those with more education had higher preference for podcast [$t_{[66]} = -2.25$, $p = 0.0281$], social media [$t_{[56.362]} = -2.30$, $p = 0.0251$], and professional in person [$t_{[52.593]} = -2.09$, $p = 0.0416$] than parents with less education.

DISCUSSION

This paper highlights the journey of parents who have a child diagnosed with SCID through newborn screening and their specific informational and emotional support needs. Our unique design allowed us to gather in-depth qualitative as well as broad quantitative data. It contributes much needed information about the challenges associated with receiving a SCID diagnosis as well as short- and long-term needs. Below, we first note the limitations to our work. Then, we discuss the results of the journey map and survey in context of the current literature. Lastly, we address implications and next steps for SCID Compass in meeting these needs.

Limitations

There are two main limitations to consider while interpreting these results. First, we used a convenience sample of parents for both the journey map activity and online survey who resided in the United States. By design, the qualitative interviews and focus group used a small, relatively heterogeneous sample of parents. Although the survey had many more participants, it was distributed through the same recruitment channels. Similarly, there were few parents from rural and medically underserved areas. Thus, our findings may not be representative of the entire SCID community, both nationally and internationally. Second, the informational and emotional support needs that parents rated were prespecified based on the data gathered in the journey map. Parents did not have an opportunity to list any additional needs. Therefore, there may be other parental needs that we did not uncover through the survey. Despite these limitations, we believe the are many noteworthy findings.

TABLE 3 | Total mean (SD) parent ratings for informational and emotional support needs and by comparison groups.

	All parents	White, non-Hispanic		Education level		Rural or immunology desert	
	Total ($n = 76$)	Yes ($n = 58$)	No ($n = 18$)	Less ($n = 36$)	More ($n = 40$)	Yes ($n = 5$)	No ($n = 71$)
Preference for material format							
Professional in person	6.04 (1.25)	5.91 (1.33)*	6.50 (0.82)	5.71 (1.51)	6.33 (0.92)*	6.09 (1.18)	5.40 (2.07)
Peer to peer	6.03 (1.41)	6.12 (1.33)	5.71 (1.69)	5.86 (1.52)	6.18 (1.32)	6.00 (1.42)	6.40 (1.34)
Print	5.59 (1.53)	5.65 (1.48)	5.38 (1.71)	5.62 (1.72)	5.56 (1.35)	5.57 (1.52)	5.80 (1.79)
Social Media	5.25 (2.09)	5.19 (2.12)	5.50 (1.99)	4.64 (2.37)	5.77 (1.68)*	5.16 (2.13)	6.470 (0.89)
Web	5.19 (1.97)	5.26 (1.95)	4.93 (2.09)	4.76 (2.25)	5.55 (1.65)	5.13 (2.00)	6.00 (1.41)
Video	4.77 (1.84)	4.62 (1.82)	5.31 (1.85)	4.51 (2.06)	5.00 (1.61)	4.72 (1.81)	5.40 (2.30)
Podcast	4.40 (1.89)	4.45 (1.89)	4.15 (1.95)	3.83 (2.12)	4.84 (1.59)*	4.32 (1.88)	6.00 (1.73)
Other	6.11 (1.05)	6.29 (1.11)	5.50 (0.71)	6.33 (0.82)	5.67 (1.53)	6.00 (1.07)	7.00 (.)

1 = Not at all to 7 = Extremely.
*$p < 0.05$.

Summary of Findings

Parents of children diagnosed with SCID through newborn screening experienced universal elements in their journey, from diagnosis through treatment and finally adjusting to the new normal once they are back home. Key informational and emotional support needs differed throughout the journey. This journey is in stark contrast to the diagnostic odyssey that is typical of clinical diagnosis (18, 19). Others also have noted a distinct journey for those diagnosed though newborn screening, suggesting there are different long-term implications for both parents, their child, as well as different relationships with healthcare providers (20). Our findings also echo the work of Rolland and colleagues that revealed the unique phases of illness as well as parental needs and adaptations that are needed along the course (21).

The highest rated information needs for families were having more information about treatment options, understanding what to expect across the SCID lifespan (i.e., knowing what the journey ahead will look like), knowing what to expect during treatment, and adjusting to life after treatment including knowing where to access to specialists and how to keep their child healthy. Although several studies have examined educational needs of healthcare providers about specific conditions or about newborn screening more broadly (22–25), fewer have focused on the information needs of parents. One study conducted focus groups with parents to better understand what they knew about newborn screening, how they wanted to learn about newborn screening, who should provide the information, and what format would be best (26). An interview study of parent who had received an abnormal metabolic newborn screening result for their child found comparable information needs to those reported in our study, including shock at receiving the test results, frustration in searching for information, and stress related to waiting for confirmatory testing and next steps for treatment (27). Other studies have reported similar information seeking behaviors by parents after a positive newborn screening result (28), such as searching online for information or reaching out to their child's healthcare provider.

Emotional support needs expressed by parents included dealing with uncertainty about their child's future, having access to other families and professional organizations, and understanding the importance of self-care and managing emotions. Earlier work has also reported on parental uncertainty following a positive newborn screen as well as ways to manage and reduce uncertainty (29–31). Common coping strategies include gathering information about the child's condition, assessing the health risk for their child, and seeking support from other. Connecting with other families who have a child with the same condition has been reported by others as well (32, 33). Parent support groups have been shown to provide many benefits, including learning practical skills from other parents; getting relevant, useful information; and obtaining reassurance about the future (34). This can lead to personal growth and empowerment as well as improved emotional well-being.

Our sample of parents who may be medically underserved, which included those who were non-White or Hispanic, those with lower education levels, or those who lived in rural areas or immunology deserts, had even higher informational and emotional support needs across a variety of areas. This included practical needs, such as accessing a specialist, as well as emotional needs, such as navigating relationships. Previous work has highlighted the needs of those who are medically underserved, with particular emphasis on those living in rural areas (35, 36). Other work has shown that education levels and race/ethnicity are social determinants of health (37, 38). The combination of living in a rural area, having lower education, and being a minority can have an additive effect which may result in poorer outcomes for children and their families.

Implications and Next Steps

Together, these findings suggest high information needs among parents about newborn screening and the implications of having a positive diagnosis for a rare disorder. Not surprisingly, this results in high levels of uncertainty and emotional distress. Thus, parents have an intense need to seek out condition-specific information in order to better understand the impact of SCID

on their child and family. Parents also desire to know what the road ahead will look like, including treatment options and how to best care for their child at home. Parents turn to trusted sources for this information, including their healthcare provider as well as other parents who have a child with SCID. Emotional support from other parents can provide hope for the future as well as a way to cope with stress and tend to parent's own mental health needs. For parents living in rural or medically underserved areas, those with lower education levels, or those from racial or ethnic minorities these needs may be exacerbated given the challenges in findings specialists who know their child's condition or other parents who have a child with SCID.

Findings from the parent needs assessment activities will serve as the foundation for creating a suite of resources for those who have a child with SCID. The materials, which will be housed on the SCID Compass web site, will be tailored to specific stages of the journey in order to meet the emotional and informational needs of parents. For example, information about SCID, the different types of SCID, newborn screening, and confirmatory testing will all be directed to parents as they are starting out their SCID journey. Next, during the Pre-treatment, Treatment, and Post-Treatment phases of the journey, parents will be provided information treatment options, the long hospital stay, and how to survive isolation. Information about how to prepare your home and future family planning will be available in the Living with SCID phase of the journey. Emotional support needs, such as where to connect with other parents, how to manage your emotions, and the importance of self-care, are applicable to many stages of the journey and thus will be posted to a dedicated section of the web site. Given that our data show that medically underserved families have increased needs, we will prioritize outreach to them.

By using a family-centered approach to gather data on unmet parental needs, we will ensure that the materials for the SCID Compass web site will be understandable, comprehensive, and useful. Our intent is that the SCID Compass web site will be a vast repository of information for families who have a child with SCID which will meet all their needs and ultimately make their journey with SCID easier.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by RTI International. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MR, LS, HP, and JB designed the overall study. MR, ML, LS, AG, KP, HP, AS, and BF designed the journey map and online survey. ML and AG analyzed the data. MR, ML, and AG drafted the manuscript. All authors critically reviewed the manuscript.

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REFERENCES

1. Buckley RH. Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Ann Rev Immunol*. (2004) 22:625–55. doi: 10.1146/annurev.immunol.22.012703.104614
2. Seth D, Ruehle M, Kamat D. Severe combined immunodeficiency: a guide for primary care givers. *Clin Pediatr*. (2019) 58:1124–7. doi: 10.1177/0009922819859867
3. 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) 312:729–38. doi: 10.1001/jama.2014.9132
4. Yee A, De Ravin SS, Elliott E, Ziegler JB. Severe combined immunodeficiency: a national surveillance study. *Pediatr Allergy Immunol*. (2008) 19: 298–38. doi: 10.1111/j.1399-3038.2007.00646.x
5. Brown L, Xu-Bayford J, Allwood Z, Slatter M, Cant A, Davies EG, et al. Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. *Blood*. (2011) 117:3243–6. doi: 10.1182/blood-2010-08-300384
6. Buckley RH. Transplantation of hematopoietic stem cells in human severe combined immunodeficiency: longterm outcomes. *Immunol Res*. (2011) 49:25–43. doi: 10.1007/s12026-010-8191-9
7. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000–2009. *N Engl J Med*. (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
8. Lipstein EA, Vorono S, Browning MF, Green NS, Kemper AR, Knapp AA, et al. Systematic evidence review of newborn screening and treatment of severe combined immunodeficiency. *Pediatrics*. (2010) 125:e1226–35. doi: 10.1542/peds.2009-1567
9. Puck JM. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. *Immunol Rev*. (2019) 287:241–52. doi: 10.1111/imr.12729
10. APHL. *Severe Combined Immunodeficiency (SCID) In-Person National Meeting*. Available online at: https://www.newsteps.org/sites/default/files/scidinpersonmeeting_meetingsummary.pdf (accessed April 26, 2020).
11. Sanchez LA, Yoo J, Rennels C, Murnane M, Mangurian C, Halley M, et al. Experiences of parents of patients with severe combined immunodeficiency disease (SCID) Identified by newborn screening: a qualitative study. *J Clin Immunol*. (2018) 38:362.

12. Dorsey MJ, Dvorak CC, Cowan MJ, Puck JM. Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. *J Allergy Clin Immunol*. (2017) 139:733–42. doi: 10.1016/j.jaci.2017.01.005
13. Pelentsov LJ, Fielder AL, Laws TA, Esterman AJ. The supportive care needs of parents with a child with a rare disease: results of an online survey. *BMC Family Pract*. (2016) 17:88. doi: 10.1186/s12875-016-0488-x
14. Richardson A. Using customer journey maps to improve customer experience. *Harv Bus Rev*. (2010) 15:2–5.
15. Miles MB, Huberman AM, Huberman MA, Huberman M. *Qualitative Data Analysis: An Expanded Sourcebook*. Thousand Oaks, CA: Sage (1994).
16. Glaser BG, Strauss AL. *Discovery of Grounded Theory: Strategies for Qualitative Research*. London: Routledge (2017). doi: 10.4324/9780203793206
17. Saunders B, Sim J, Kingstone T, Baker S, Waterfield J, Bartlam B, et al. Saturation in qualitative research: exploring its conceptualization and operationalization. *Qual Quant*. (2018) 52:1893–907. doi: 10.1007/s11135-017-0574-8
18. Bailey DB Jr, Beskow LM, Davis AM, Skinner D. Changing perspectives on the benefits of newborn screening. *Ment Retard Dev Disabil Res Rev*. (2006) 12:270–9. doi: 10.1002/mrdd.20119
19. Borte S, von Döbeln U, Hammarström L. Guidelines for newborn screening of primary immunodeficiency diseases. *Curr Opin Hematol*. (2013) 20:48–54. doi: 10.1097/MOH.0b013e32835a9130
20. McKay K, Wilcken B. Newborn screening for cystic fibrosis offers an advantage over symptomatic diagnosis for the long term benefit of patients. *Paediatr Respir Rev*. (2008) 9:290–4. doi: 10.1016/j.prrv.2008.09.004
21. Rolland JS, Walsh F. Facilitating family resilience with childhood illness and disability. *Curr Opin Pediatr*. (2006) 18:527–38. doi: 10.1097/01.mop.0000245354.83454.68
22. Comeau AM, Parad R, Gerstle R, O'Sullivan BP, Dorkin HL, Dovey M, et al. Challenges in implementing a successful newborn cystic fibrosis screening program. *J Pediatr*. (2005) 147:S89–93. doi: 10.1016/j.jpeds.2005.08.006
23. Faulkner LA, Feuchtbaum LB, Graham S, Bolstad JP, Cunningham GC. The newborn screening educational gap: what prenatal care providers do compared with what is expected. *Am J Obstet Gynecol*. (2006) 194:131–7. doi: 10.1016/j.ajog.2005.05.075
24. Kemper AR, Trotter TL, Lloyd-Puryear MA, Kyler P, Feero WG, Howell RR. A blueprint for maternal and child health primary care physician education in medical genetics and genomic medicine: recommendations of the United States secretary for health and human services advisory committee on heritable disorders in newborns and children. *Genet Med*. (2010) 12:77–80. doi: 10.1097/GIM.0b013e3181cb78fa
25. Bellcross C, Harmond L, Floyd-Browning P, Singh R. infrastructure and educational needs of newborn screening short-term follow-up programs within the southeast regional newborn screening & genetics collaborative: a pilot survey. *Healthcare*. (2015) 3:964–72. doi: 10.3390/healthcare3040964
26. Davis TC, Humiston SG, Arnold CL, Bocchini JA, Bass PF, Kennen EM, et al. Recommendations for effective newborn screening communication: results of focus groups with parents, providers, and experts. *Pediatrics*. (2006) 117(Suppl. 3):S326–40. doi: 10.1542/peds.2005-2633M
27. DeLuca JM, Kearney MH, Norton SA, Arnold GL. Parents' experiences of expanded newborn screening evaluations. *Pediatrics*. (2011) 128:53–61. doi: 10.1542/peds.2010-3413
28. Dillard JP, Shen L, Robinson JD, Farrell PM. Parental information seeking following a positive newborn screening for cystic fibrosis. *J Health Commun*. (2010) 15:880–94. doi: 10.1080/10810730.2010.522226
29. Price Dillard JA, Carson CL. Uncertainty management following a positive newborn screening for cystic fibrosis. *J Health Commun*. (2005) 10:57–76. doi: 10.1080/10810730590904580
30. Goldenberg AJ, Comeau AM, Grosse SD, Tanksley S, Prosser LA, Ojodu J, et al. Evaluating harms in the assessment of net benefit: a framework for newborn screening condition review. *Matern Child Health J*. (2016) 20:693–700. doi: 10.1007/s10995-015-1869-9
31. Tluczek A, Koscik RL, Farrell PM, Rock MJ. Psychosocial risk associated with newborn screening for cystic fibrosis: parents' experience while awaiting the sweat-test appointment. *Pediatrics*. (2005) 115:1692–703. doi: 10.1542/peds.2004-0275
32. Boyse KL, Gardner M, Marvicsin DJ, Sandberg DE. "It was an overwhelming thing": Parents' needs after infant diagnosis with congenital adrenal hyperplasia. *J Pediatr Nurs*. (2014) 29:436–41. doi: 10.1016/j.pedn.2014.01.007
33. Siklos S, Kerns KA. Assessing need for social support in parents of children with autism and down syndrome. *J Autism Dev Disord*. (2006) 36:921–33. doi: 10.1007/s10803-006-0129-7
34. Ainbinder JG, Blanchard LW, Singer GH, Sullivan ME, Powers LK, Marquis JG, et al. A qualitative study of parent to parent support for parents of children with special needs. Consortium to evaluate parent to parent. *J Pediatr Psychol*. (1998) 23:99–109. doi: 10.1093/jpepsy/23.2.99
35. Marcin JP, Ellis J, Mawis R, Nagrampa E, Nesbitt TS, Dimand RJ. Using telemedicine to provide pediatric subspecialty care to children with special health care needs in an underserved rural community. *Pediatrics*. (2004) 113:1–6. doi: 10.1542/peds.113.1.1
36. Heath B, Salerno R, Hopkins A, Hertzig J, Caputo M. Pediatric critical care telemedicine in rural underserved emergency departments. *Pediatr Crit Care Med*. (2009) 10:588–91. doi: 10.1097/PCC.0b013e3181a63eac
37. Blane D. Social determinants of health–socioeconomic status, social class, and ethnicity. *Am J Public Health*. (1995) 85:903–5. doi: 10.2105/AJPH.85.7.903
38. Fuchs VR. Social determinants of health: caveats and nuances. *JAMA*. (2017) 317:25–6. doi: 10.1001/jama.2016.17335

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Second-Tier Next Generation Sequencing Integrated in Nationwide Newborn Screening Provides Rapid Molecular Diagnostics of Severe Combined Immunodeficiency

*Janne Strand[1], Kiran Aftab Gul[2], Hans Christian Erichsen[3,4], Emma Lundman[1], Mona C. Berge[1], Anette K. Trømborg[1], Linda K. Sørgjerd[1], Mari Ytre-Arne[1], Silje Hogner[1], Ruth Halsne[1,5], Hege Junita Gaup[1], Liv T. Osnes[6], Grete A. B. Kro[7], Hanne S. Sorte[8], Lars Mørkrid[9], Alexander D. Rowe[1], Trine Tangeraas[1,3], Jens V. Jørgensen[1,3], Charlotte Alme[10], Trude E. H. Bjørndalen[11], Arild E. Rønnestad[3], Astri M. Lang[3], Terje Rootwelt[3,4], Jochen Buechner[10], Torstein Øverland[3], Tore G. Abrahamsen[3,4], Rolf D. Pettersen[1] and Asbjørg Stray-Pedersen[1,3]**

[1] Norwegian National Unit for Newborn Screening, Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway, [2] Paediatric Research Institute, Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway, [3] Department of Paediatrics, Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway, [4] Division of Paediatric and Adolescent Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway, [5] Department of Forensic Biology, Oslo University Hospital, Oslo, Norway, [6] Department of Immunology and Transfusion Medicine, Oslo University Hospital, Oslo, Norway, [7] Department of Microbiology, Oslo University Hospital, Oslo, Norway, [8] Department of Medical Genetics, Oslo University Hospital, Oslo, Norway, [9] Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway, [10] Department of Paediatric Haematology, Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway, [11] Department of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway

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Jolan Eszter Walter,
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Hamoud Al-Mousa,
King Faisal Specialist Hospital & Research Centre, Saudi Arabia
Catharina Schuetz,
Universitätsklinikum Carl Gustav Carus, Germany
Attila Kumanovics,
Mayo Clinic, United States

***Correspondence:**
Asbjørg Stray-Pedersen
astraype@ous-hf.no

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Severe combined immunodeficiency (SCID) and other T cell lymphopenias can be detected during newborn screening (NBS) by measuring T cell receptor excision circles (TRECs) in dried blood spot (DBS) DNA. Second tier next generation sequencing (NGS) with an amplicon based targeted gene panel using the same DBS DNA was introduced as part of our prospective pilot research project in 2015. With *written* parental consent, 21 000 newborns were TREC-tested in the pilot. Three newborns were identified with SCID, and disease-causing variants in *IL2RG, RAG2,* and *RMRP* were confirmed by NGS on the initial DBS DNA. The molecular findings directed follow-up and therapy: the *IL2RG*-SCID underwent early hematopoietic stem cell transplantation (HSCT) without any complications; the leaky *RAG2*-SCID received prophylactic antibiotics, antifungals, and immunoglobulin infusions, and underwent HSCT at 1 year of age. The child with *RMRP*-SCID had complete Hirschsprung disease and died at 1 month of age. Since January 2018, all newborns in Norway have been offered NBS for SCID using 1st tier TRECs and 2nd tier gene panel NGS on DBS DNA. During the first 20 months of nationwide SCID screening an additional 88 000 newborns were TREC tested, and four new SCID cases were identified. Disease-causing variants in *DCLRE1C*, *JAK3, NBN,* and *IL2RG* were molecularly confirmed on day 8, 15, 8 and 6, respectively after birth, using the initial NBS blood spot. Targeted gene panel NGS integrated into the NBS algorithm rapidly delineated the specific molecular diagnoses and provided information useful for management, targeted therapy and follow-up i.e., X rays and CT scans were avoided

in the radiosensitive SCID. Second tier targeted NGS on the same DBS DNA as the TREC test provided instant confirmation or exclusion of SCID, and made it possible to use a less stringent TREC cut-off value. This allowed for the detection of leaky SCIDs, and simultaneously reduced the number of control samples, recalls and false positives. Mothers were instructed to stop breastfeeding until maternal *cytomegalovirus* (CMV) status was determined. Our limited data suggest that shorter time-interval from birth to intervention, may prevent breast milk transmitted CMV infection in classical SCID.

Keywords: SCID - severe combined immunodeficiency, newborn SCID screening, NGS - next generation sequencing, severe T-cell immunodeficiency, TREC analysis

INTRODUCTION

Early diagnosis is important in severe combined immunodeficiency (SCID). Targeted treatment, molecularly adjusted preconditioning, as well as avoiding infections such as *cytomegalovirus* (CMV) before undergoing hematopoietic stem cell transplantation (HSCT), improves overall prognosis (1–4). Newborn screening (NBS) for SCID was first introduced in the United States in 2008[1]. SCID became part of the US core recommended uniform screening panel (RUSP) in 2010, and since December 2018 all US states have implemented SCID screening as part of their NBS program[2]. Nationwide SCID screening was implemented in Taiwan 2012 (5, 6), and Israel Oct 2015 (6, 7). In Europe, universal SCID screening started in the region of Catalonia in Spain (Jan 2017) (8), Iceland (2017), Switzerland (Jan 2019), Sweden and Germany (Aug 2019) proceeded by pilots (9–11)[3], and Denmark (Feb 2020) (12). And hospital pilots have been performed (13), or are ongoing in European countries and regions such as France (14), Finland, Poland, Italy, and the Netherlands (15, 16).

SCID and other T cell lymphopenias can be identified during newborn screening (NBS) by measuring T cell receptor excision circles (TRECs) in DBS DNA (17)[4]. Common SCID screening algorithms include quantification of TRECs as 1st tier, sometimes followed by a repeated control blood sample from the baby (18). Based on each laboratory's cut-off values for TRECs, 1–10 per 10,000 newborn babies are reported as screening positives (18, 19). These are referred to hospital and undergo clinical pediatric evaluation and venous blood sampling for flow cytometric quantification of lymphocyte subsets in order to reach a diagnosis.

Disease causing variants in more than 40 different genes may cause SCID or severe T cell deficiency (20). Knowledge of the specific molecular genetic cause of the immunodeficiency may direct individualized therapy and define the preconditioning regimen for the lifesaving HSCT or thymic transplantation (21). The molecular detection rate for SCID is high, in contrast to other primary immunodeficiencies (22, 23). Atypical, variant and "leaky" SCID are characterized by diminished T cell immunity with low, but non-zero TRECs, with a few exceptions (*ZAP70, ORAI1*, or MHC class II deficiencies) (24). Atypical SCIDs are caused by pathological, but often less deleterious, variants in the same genes as classical SCIDs (4, 24, 25). Therefore, we chose to test the utility of including molecular testing *within* the NBS laboratory's algorithm for SCID. Others have in a retrospective study reported the feasibility of a similar strategy (26), while of ethical reasons we chose a prospective approach. Furthermore, we wanted to establish a pipeline for reliable and rapid SCID screening which could be implemented in the ordinary NBS program.

METHODS

The *prospective* part of the study "NBS for SCID" was designed as a research project and required *written* consent for participation. Parents of newborns born in six selected hospitals in Norway were offered SCID screening of their children. The SCID testing was performed using a single 3.2 mm punch from the same dried blood spot (DBS) filter card (Perkin Elmer 226 specimen collection device or 903 cards from Eastern Business Forms) as the other tests in the ordinary NBS program. When the written consent was received at the national screening unit, identifiers were manually checked and registered. Corresponding DBS samples were selected from the biobank for punching, DNA extraction, and further molecular testing. This pilot study was approved by the Regional Ethical Committee (REC number 2014/128, NBS for SCID) and was conducted in the period Sept 22nd 2015 to Dec 31st 2017. In parallel, we performed a *retrospective* study using samples from patients with primary immunodeficiencies (PIDs) with known and unknown molecular diagnoses to evaluate our methods, establish cut-off levels, and to determine methodological sensitivity and specificity for these disorders (SCID and T cell lymphopenias). It also enabled us to explore the utility and efficacy of 2nd tier NGS integrated in NBS for SCID. The *retrospective* studies were part of the project *Identification of genetic causes of primary immunodeficiency and immunodysregulation using high-throughput sequencing* (REC number 2014/1270) (22). This protocol allowed for testing of individuals with clinically suspected SCID and PID but born at hospitals not included in the pilot. When SCID screening was implemented nationwide in the Norwegian NBS program

[1]https://primaryimmune.org/idf-advocacy-center/idf-scid-newborn-screening-campaign
[2]https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html
[3]https://www.g-ba.de/beschluesse/3586/
[4]Clinical and Laboratory Standards Institute (CLSI). Newborn blood spot screening for severe combined immunodeficiency by measurement of T-cell receptor excision circles; Approved guideline. CLSI NBS06-A: 1ED 2013.

TABLE 1 | New SCIDs and severe T cell deficiencies identified on newborn screening.

Patient ID	Pilot project			National screening					
	SCID_1	**SCID_2**	**SCID_3**	**SCID_4**	**SCID_5**	**SCID_6**	**SCID_7**	**CID_1**	**CID_2**
Symptoms at time of diagnosis	Healthy	Healthy	Skeletal dysplasia, total Hirschprung	Healthy	Healthy	Microcephaly SGA	Healthy	Healthy, Thymic aplasia	Hydrops fetalis, congenital, heart disease, arthrogryposis
Gender	Male	Male	Female	Male	Male	Female	Male	Female	Male
GA w	42	41	37	40	39	40	Male	41	28
BW g	3,592	3,588	2,152	3,618	2,515	2,855	41	4,065	1,330
Mean TRECs/μl	0.48	9.7	0	0	2	1.2	0	11.3	2.12
TREC results avaliable	Day 16	Day 12	Day 11	Day 4	Day 13	Day 6	Day 4	Day 8	Day 4
Age at molecular diagnosis	Day 22	Day 17	After death	Day 8	Day 15	Day 8	Day 6	NA	Prenatal test
Gene	*IL2RG*	*RAG2*	*RMRP*	*DCLRE1C*	*JAK3*	*NBN*	*IL2RG*	none	Trisomy 21
SNV/CNV	c.[359dupA];[0]	c.[1367C>T] HOM	n.[71A>G] HOM	c.[82C>G] HOM	c.[1767C>T]; [2077C>A]	c.[657_661del] HOM	c.[371T>C];[0]	No finding	47, XY, +21
protein	p.Glu121Glyfs*47	p.Ala456Val	NA	p.Ala28Pro	p.(Gly589=); (Pro693Thr)	p.Lys219Asnfs*16	p.Leu124Pro	NA	NA
Refseq	NM_000206.2	NM_000536.3	NR_003051.3	NM_ 001033855.2	NM_000215.3	NM_002485.4	NM_000206.2	NA	NA
Methods	PIDv2 gene panel	PIDv2 gene panel	PIDv2 gene panel	NBSv2 gene panel	NBSv2 gene panel	NBSv2 gene panel	NBSv2 gene panel	NBSv2 and PIDv2, clinical WES trio and aCGH	Prenatal: Trisomy test and aCGH Postnatal: NBSv2 and clinical WES
Outcome	HSCT, Successful	HSCT, Successful	Deceased 1 month old	HSCT, Successful	HSCT, Successful	Antimicrobial prophylaxis, HSCT considered	HSCT, Successful	Clinical follow-up only, thymus transplantation considered	Deceased 1 month old

aCGH, array comparative genomic hybridization chromosomal/chromosomal microarray; BW, Birth weight; CNV, Copy number variation; g, gram; GA, Gestational age; NA, Not applicable; NBS, Newborn screening; NBSv2, Newborn screening gene panel version 2; PIDv2, Primary immunodeficiency research panel version 2; PID, Primary immunodeficiency; RefSeq, The National Center for Biotechnology Information Reference Database; SCID, Severe combined immunodeficiency; SNV, Single nucleotide variant; TREC, T-cell receptor excision circles; Trio-test; testing the child and both parents in comparison; WES, whole exome sequencing; w, week.

(January 1st 2018), parental consent was based on *informed* (not written) consent. The parents of the children with SCID and T cell deficiencies identified in the national screening program, in the prospective pilot, and in the retrospective study (**Tables 1**–**4**), have given written consent to the publication of the medical information included about their child.

In many countries NBS is mandatory, while in Norway NBS is voluntary and based on informed but not written, consent from one of the parents, usually the mother. The parental consent has in practice been obtained similar to the "informed compliance method" described by Kelly et al. (27). Historically, it has been a high (99.9%) participation in the Norwegian NBS program (28). Blood samples from a heel prick of newborn babies are collected on filter cards between 48 and 72 h after birth and sent by overnight express mail. One centralized laboratory, The Norwegian National Newborn Screening Unit performs all analyses. The screening unit is located at the largest University hospital and main pediatric referral center in the country.

DNA EXTRACTION

DNA was extracted from a 3.2 mm punch of the DBS sample card collected in the ordinary routine NBS program. Samples were punched from the filter card using a Panthera-Puncher 9 (Perkin Elmer, Turku, Finland). The manual method for DNA extraction from filter card blood is modified from Heath et al. (29), and published in detail elsewhere (28). Briefly, the punch was washed with 150 μL DNA Elution solution Qiagen (S2) at 60°C with continuous shaking, followed by elution in 100 μL S2 at 99.5°C for 30 min. One 3.2 mm punch contains on average 3 μL blood, and the extraction method described yields approximately 30 ng DNA from each punch.

TABLE 2 | New severe PIDs, debut 3–6 months, not picked up on newborn screening since born outside pilot test region.

Patient ID	TRECs and PIDv2 panel testing on the original DBS and the new sample					
	PID_1	**PID_2**	**PID_3**	**PID_4**	**PID_5**	**PID_6**
Year	2016	2016	2017	2017	2017	2017
Symptoms at time of diagnosis	SGA, microcephaly, transient lymphopenia, persistent neutropenia and low number of platelets	SGA, microcephaly, failure to thrive, pigment patches skin	Heart defect, choanal atresia, coloboma, infections	Heart defect, prematurity, Downs syndrome	Heart defect, dysmorphic features	Infections, failure to thrive
Gender	Female	Male	Male	Male	Male	Female
GA w	35	36	36	33	34	40
BW g	1,920	1,534	2,445		1,570	3,430
TRECs/μl at birth	4.69	1.9	0	9.7	15.7	71.8
Repeated TRECs/ul (age)	23.8 (3 months) 0 (12 months)	0 (4 months)	0 (1.5 months)	0 (2 months)	NA	12.2 (6.8 months)
Gene	unknown	unknown	*CHD7*	Trisomy 21	*TBX1*	*IKZF1*
SNV/CNV	NA	NA	c.[5833C>T];[=]	47, XY, +21	22q11.21 del	c.[476A<G];[=]
Protein	NA	NA	p.Arg1945*	NA	NA	p.Asn159Ser
Refseq	NA	NA	NM_017780.3	NA	NA	NM_006060.4
Methods	PIDv2 gene panel, WES/WGS trio	PIDv2 gene panel, WES/WGS trio	Sanger	Trisomy test	MLPA, Trisomy test	PIDv2 gene panel
Treatment and outcome	Deceased at 1 ½ years of age	HSCT, Successful	Prophylactic antibiotics, antifungal, antiviral and Sclg therapy	Heart surgery. Lymphopenia 1.9×10^9/L at 1 years of age, but no recurrent infections	Prophylactic antibiotics	HSCT, Successful

aCGH, array comparative genomic hybridization chromosomal/chromosomal microarray; BW, Birth weight; CNV, Copy number variation; g, gram; GA, Gestational age; HGNC, The HUGO Gene Nomenclature Committee; HSCT, Hematopoietic Stem Cell Transplantation; MLPA, multiplex ligation-dependent probe amplification; NA, Not applicable; NBS, Newborn screening; ND, Not detected; PIDv2, Primary immunodeficiency research panel version 2; PIDs, Primary immunodeficiencies; RefSeq, The National Center for Biotechnology Information Reference Database; SCID, Severe combined immunodeficiency; Sclg, Subcutaneous Immunoglobulin; SGA, Small for gestational age; SNV, Single nucleotide variant; TREC, T-cell receptor excision circles; Trio-test; testing the child and both parents in comparison; WES, whole exome sequencing; WGS, whole genome sequencing w, week.
Resources: Gene names according to HGNC, https://www.genenames.org/.
Gene variant nomenclature according to the HGVS recommendations, http://www.HGVS.org/varnomen.

TRECs AND β-ACTIN

TRECs and β-actin levels were quantified by qPCR on ViiA7 and QuantStudio 7 (Applied Biosystems/Thermo Fisher Scientific, CA, USA) real-time PCR systems. TREC and β-actin were analyzed in a final volume of 20 μL containing 10 μl PerfeCTa qPCR Toughmix (2x, Quanta Biosciences), 0.8 μl BSA (10 mg/ml) and the following primer sequences: 0.5 μL TREC Forward 5′-CAC ATC CCT TTC AAC CAT GCT-3′ (20 μM), 0.5 μL TREC Reverse 5′-GCC AGC TGC AGG GTT TAG G-3′ (20 μM), 0.2 μL TREC Probe: 5′-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-3′-TAMRA (15 μM), or ACTB primers: 0.5 μL β-actin Forward 5′-ATT TCC CTC TCA GGC ATG GA-3′ (10 μM), 0.5 μL β-actin Reverse 5′-CGT CAC ACT TCA TGA TGG AGT TG-3′ (10 μM), 0.2 μL β-actin Probe: 5′-FAM-GTG GCA TCC ACG AAA CTA-3′-TAMRA (15 μM). To the TREC assay 8 μl DNA was added. To the β-actin assay 4 μL DNA and 4 μl nuclease-free water were added. The reactions were carried out with an initial step at 50°C for 2 min, a denaturation stage at 95°C for 10 min followed by 45 cycles at 95°C for 30 s, and 60°C for 60 s. A calibration curve was created from a TREC plasmid generated and kindly provided by Douek et al. (30). TREC plasmid concentration was determined by Nanodrop (Spectrophotometer ND-1000), and an 8 point standard curve was constructed after 2-fold serial dilutions in dilution solution (Qiagen Generation solution 2 containing tRNA 50 ng/μL). All the qPCR assessments fulfilled the quality requirements of similar slopes and with R^2 values > 0.975. β-actin was used as a reference gene to assure adequate DNA extraction, and β-actin was only analyzed in samples with TREC values below cut-off values. Our TREC values per μl were based on the assumption that a 3.2 mm punch contains 3 μL of blood. Cut-off value was set to 25 TRECs/μL, and only samples below this initial cut-off were re-run. Samples with normal

TABLE 3 | Retrospective TRECs and NGS testing in known PIDs using DNA from the original newborn screening DBS.

Sample ID	Individuals with known SCID or severe T-cell deficiency									Other PIDs	
	KID_1	KID_2	KID_3	KID_4	KID_5	KID_6	KID_7	KID_8	KID_9	KID_10	KID_11
Year	2012	2010	2010	2015	2012	2014	2015	2006	2009	2009	2012
Gender	Male	Female	Female	Female	Female	Male	Male	Female	Male	Male	Female
GA w	37	34	38,5	42	40	42	39	38	39	40	38
BW g	2,954	1,999	2,618	3,360	4,135	4,420	2,484	NA	3,445	3,775	2,950
TRECs/μl	0	0	0	0	11	0	0	0	0	25.5	60
Gene	*IL2RG*	*LIG4*	*IL7R*	*ADA*	*PGM3*	*JAK3*	*TBX1*	*TBX1*	*TBX1*	*IKZF1*	*RECQL4*
SNV/CNV	c.[924+5G>A]; [0]	c.[1341G>T]; [482delC]	c.[707-2A>G] HOM	c.[7C>T] HOM	c.[737A>G] HOM	c.[1837C>T]; [1695C>A]	22q11.21 del	22q11.21 del	22q11.21 del	c.1618388_589 +2308del (16.8 kb del exons 4-5)	c.[2269C>T]; ND (22)
Protein	Splice defect	p.Trp447Cys; p.Ala161Valfs*6	Splice defect	p.Gln3*	p.Ala246Gly	p.Arg613*; p.Cys565*	loss	loss	loss	Inframe deletion	p.Gln757*
RefSeq	NM_ 000206.2	NM_ 002312.3	NM_ 002185.3	NM_ 000022.2	NM_ 015599.2	NM_ 000215.3	NM_ 080647.1	NM_ 080647.1	NM_ 080647.1	NM_ 006060.5	NM_ 004260.3
Panel	PIDv1	PIDv1	PIDv1	PIDv2	PIDv2	PIDv1	PIDv2	PIDv2	PIDv2	PIDv2	PIDv1 and PIDv2

BW, Birth weight; CNV, Copy number variation; DBS, dried blood spot; del, deletion; g, gram; GA, Gestational age; HOM, Homozygous; HGNC, The HUGO Gene Nomenclature Committee; NBS, Newborn screening; ND, Not detected; NGS, Next generation sequencing; PIDs, Primary immunodeficiencies; PIDv2, Primary immunodeficiency research panel version 2; RefSeq, The National Center for Biotechnology Information Reference Database; SCID, Severe combined immunodeficiency; SNV, Single nucleotide variant; TREC, T-cell receptor excision circles; w, weeks.

Resources: Gene names according to HGNC, https://www.genenames.org/; Gene variant nomenclature according to the HGVS recommendations, http://www.HGVS.org/varnomen.

Extended information available in ***Supplemental Table S4****.*

TABLE 4 | Retrospective TRECs and NGS testing in known PIDs using DNA from the original newborn screening DBS.

Individuals with known ataxia telangiectasia

Sample ID	AT_1.1	AT_1.2	AT_2	AT_3	AT_4	AT_5	AT_6	AT_7	AT_8
Year	2008	2011	2010	2010	2011	2011	2013	2014	2016
Gender	Male	Male	Female	Female	Male	Male	Male	Female	Female
GA w	36	39	40	40	41	41	39	40	40
BW g	3,015	3,650	3,196	3,570	2,815	4,180	3,020	3,490	2,959
TRECs/μl	3.9	2.8	15.7	7	21.7	92.8	67.2	202.7	27.2
Gene, RefSeq					*ATM*, NM_000051.3				
SNV/CNV	c.[6047A>G] HOM	c.[6047A>G] HOM	c.[3245_3247delATC insTGAT] HOM	c.[3245_3247delATC insTGAT];[6679C>T]	c.[3245_3247delATC insTGAT] HOM	c.[1564_1565delGA]; [9023G>A]	c.[3245_3247delATC insTGAT];[5712dupA]	c.[3245_3247delATC insTGAT];[8030A>G]	c.[5932G>T]; [9126delC]
protein	p.Asp2016Gly	p.Asp2016Gly	p.(His1082Leufs*14)	p.(His1082Leufs*14); p.Arg2227Cys	p.(His1082Leufs*14)	p.Glu522Ilefs*43; p.Arg3008His	p.(His1082Leufs*14); p.Ser1905Ilefs*25	p.(His1082Leufs*14); p.Tyr2677Cys	p.Glu1978*; p.Asn3044Ilefs*31
Panel	PIDv2	PIDv2	PIDv2	PIDv2	PIDv2	PIDv2	NBSv2	NBSv1	PIDv2

BW, Birth weight; CNV, Copy number variation; DBS, dried blood spot; del, deletion; del, deletion; dup, duplication; g, gram; GA, Gestational age; HOM, Homozygous; HGNC, The HUGO Gene Nomenclature Committee; ins, insertion; NBSv1, Newborn screening gene panel version 1; NGS, Next generation sequencing; PIDv2, Primary immunodeficiency research panel version 2; RefSeq, The National Center for Biotechnology Information Reference Database; SNV, Single nucleotide variant; TREC, T-cell receptor excision circles; w, week.

Resource: Gene variant nomenclature according to the HGVS recommendations, http://www.HGVS.org/varnomen.

levels of β-actin (≥ 5,000/μL) and TREC levels in duplicate below 20 TRECs/μL were NGS gene panel tested, dependent on gestational age (GA), birth weight (BW), and information in the baby's medical record (The algorithms are presented in **Figures 1A,B**, **2**).

NGS GENE PANELS

For the specific SCID gene panel test used in the retrospective study, DBS derived DNA (1.5–1.8 ng) was analyzed using the Ion AmpliSeq library kit with the Thermo Fisher predesigned gene panel PIDv1 containing 266 primary immunodeficiency disease genes, and then sequenced on a benchtop ION-PGM (Thermo Fisher Scientific). The PIDv1 gene panel was upgraded at our request by the vendor to PIDv2 with better coverage of certain critical regions such as *ADA* exon 1 (See Discussion). PIDv2 was used as the 2nd tier DNA assessment in the prospective pilot study. The annotated variant calling file (vcf) was filtered in Ion Reporter™ Software to show only variants in the genes relevant for SCID and severe T cell deficiencies. For copy number variant (CNV) detection in the NGS data the Ion Reporter's Confident CNVs-CNVs Only filter was used, and manual visualization of the genes of particular interest. The BAM files were visualized in Integrative Genomics Viewer (IGV) (31) and Alamut Visual (v.2.11, Interactive Bioinformatics, France). Variant evaluation was performed according to the ACMG guidelines (32). The assumed pathogenic gene variants identified by NGS were confirmed using Sanger sequencing, and segregation testing of the parents was performed. In a further development we used the Ion AmpliSeq On-Demand pipeline (Thermo Fisher Scientific) for customized design and synthesis of an NBS-dedicated multiplexed gene panel. This gene panel includes genes for SCID, severe T cell deficiencies and congenital bone marrow failure, genes for the metabolic disorders included in our national NBS program and in addition genes for the US' recommended uniform screening program (RUSP) disorders and their differential diagnoses. This NBS-NGS core panel was designed as a universal newborn screening panel, to be used in the nationwide SCID screening and as 2nd tier DNA testing in general in the NBS program, and the 160-gene list for NBSv1 and upgraded version NBSv2 and NBSv3 containing 186 genes (60 related to SCID/CID/T cell deficiency) are provided as **Supplemental Table S7** in the Supplementary Material. Primers have been designed to provide amplicons (average 200 bp) with 99% coverage of coding sequence and a minimum of 10 bp flanking regions of associated introns.

FOLLOW-UP OF SCREENING POSITIVES

Samples with the lowest TRECs (≤5/μL) were regarded as screening positive and immediately reported. For the others with intermediate low TRECs (5–20/μl) only those with molecular confirmation of disease (defined as ACMG class 4 or 5, or a class 3 variant in trans with a 4–5) (32), were regarded as screening positives. After a phone call to parents, the screening positive newborns were admitted to the pediatric immunologists at Oslo University Hospital who coordinated further clinical and laboratory work-ups of the children and serology testing of the mother, according to the agreed protocol: The child was isolated and hygiene advice given to the family awaiting the results of the clinical work-up and new blood samples. Breastfeeding was discontinued (milk production sustained by breast-pumping) until the maternal CMV status was determined as described below. After verification of the SCID with flow cytometry, prophylaxis with antibiotics, antifungals, antivirals, and immunoglobulin infusions was initiated awaiting HSCT.

FLOW CYTOMETRY

Flow cytometric assessments with quantification of absolute counts for T cell (CD3, CD4, CD8), B cell (CD19), and NK cell (CD16/CD56) subsets were performed in EDTA-blood from the child (See **Supplemental Tables S1**, **S5**). Absolute counts were analyzed using Multitest 6-color TBNK including Truecount beads according to the manufacturers' instructions (BD Bioscience, San Jose, CA). Further analyses of T cell subpopulations were done to identify recent thymic emigrants (RTE), naïve and memory CD4+ T cells, and if enough sample material was available, additional subpopulations of T and B cells were analyzed. EDTA blood was incubated with optimally titrated antibodies for 15 min at room temperature, followed by erythrocyte lysis using BD FACS Lysing Solution (Beckman Dickinson, San Jose, CA). For B cell analysis, the blood samples were washed twice before incubation with antibodies. Data acquisition was performed on a Gallios Flow Cytometer (Beckman Coulter, San Diego, CA). The following antibodies were used: CD31, CD45RO, CD28, CD45RA, CD127, CD19, CD27; Becton Dickinson, CD4, CD8, CD3, CD25, CD38, IgM, IgD; Beckman Coulter, TCR alfa/beta, CD21; R&D Systems (Minneapolis, MN), CXCR5; eBioscience (San Diego, CA), CD45; Invitrogen (Waltham, MA), CD27; Dako (Glostrup, Denmark). T cells were gated as CD3+ and further as naive CD4+ (CD4+, CD45RA+), recent thymic emigrants (CD4+, CD45RA+, CD31+), CD4+ memory (CD4+, CD45RO+), follicular like CD4+ (CD4+, CD45RO+, CCR5+), regulatory T cells (CD4+, CD25++, CD127–), naive CD8+ (CD8+, CD27+, CD28+), CD8+ early effector memory (CD8+, CD27+, CD28–), CD8+ late effector memory (CD8+, CD27–, CD28–). B cells were gated as CD19+ and further subclassified as naive (IgD+, IgM+, CD27–), IgM memory (CD27+, IgD+, IgM+), class switched (CD27+, IgM–, IgD–), plasmablasts (CD19+dim, CD27++, CD38++), transitional (IgM++, CD38++, CD24+), and CD21 low B cells (CD38 low, CD21 low).

CMV ANALYSES

CMV serostatus of the mother was investigated immediately after positive SCID screening not awaiting the molecular confirmation when TREC was ≤5/μL. The Abbott Architect chemiluminescent microparticle immunoassay (CMIA) (Abbott Laboratories, USA) was used for the qualitative detection of IgM and IgG antibodies to CMV. If maternal serology was CMV positive, defined as IgM

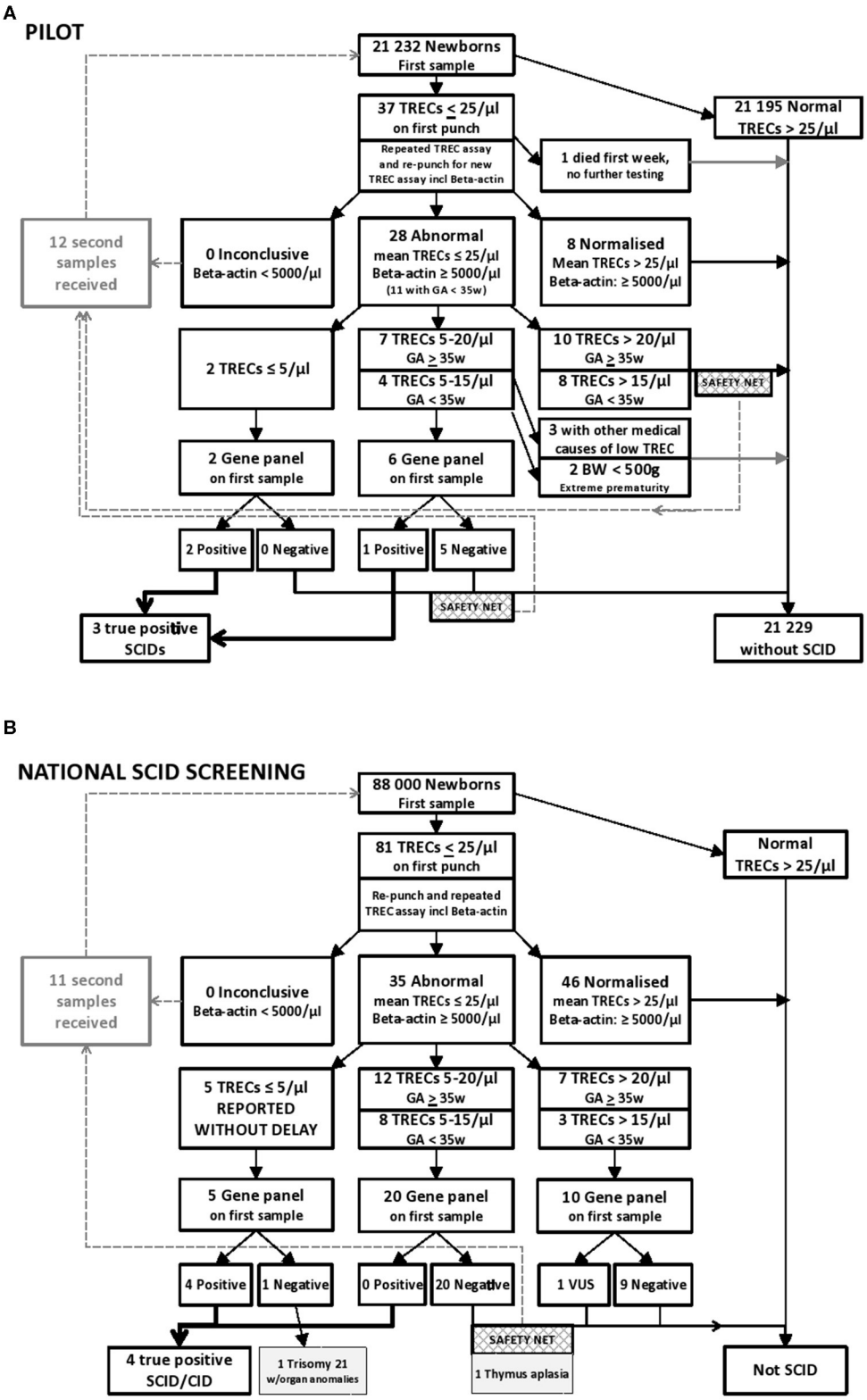

FIGURE 1 | (A) Results from the prospective pilot study with NGS gene panel testing integrated in the NBS laboratory algorithm for SCID and other T cell lymphopenias. Samples below 25 TRECs/µL were re-run and re-punched for one new TREC analysis. Samples with normal levels of β-actin (≥ 5,000/µL) and mean

(Continued)

FIGURE 1 | TREC levels below 20 TRECs/μL were NGS gene panel tested, dependent on gestational age (GA), birth weight (BW), and information in the baby's medical record. Out of the samples tested in the prospective pilot project, three individuals were identified with SCID. One of them had low intermediate TREC values, consistent with a "leaky" SCID. **(B)** Results from 20 months nationwide screening with rapid NGS gene panel testing integrated in the NBS laboratory algorithm for SCID and other T cell lymphopenias. Samples below 25 TRECs/μL were re-punched and TREC analyses repeated twice on DNA from the new punch. Samples with normal levels of β-actin (≥ 5,000/μL) and mean TREC levels below 25 TRECs/μL were NGS gene panel tested. Out of the samples tested in this nationwide screening between January 2018 to August 2019, 5 had TRECs below 5/μL, and four individuals were identified with severe primary immunodeficiency. The last one had Trisomy 21 with multiple anomalies. As a "safety net" a second DBS sample was requested if NGS was negative when TREC levels were below 20 TRECs/μL (15 TRECs/μL for prematures), which allowed for detection of one individual with congenital thymic aplasia.

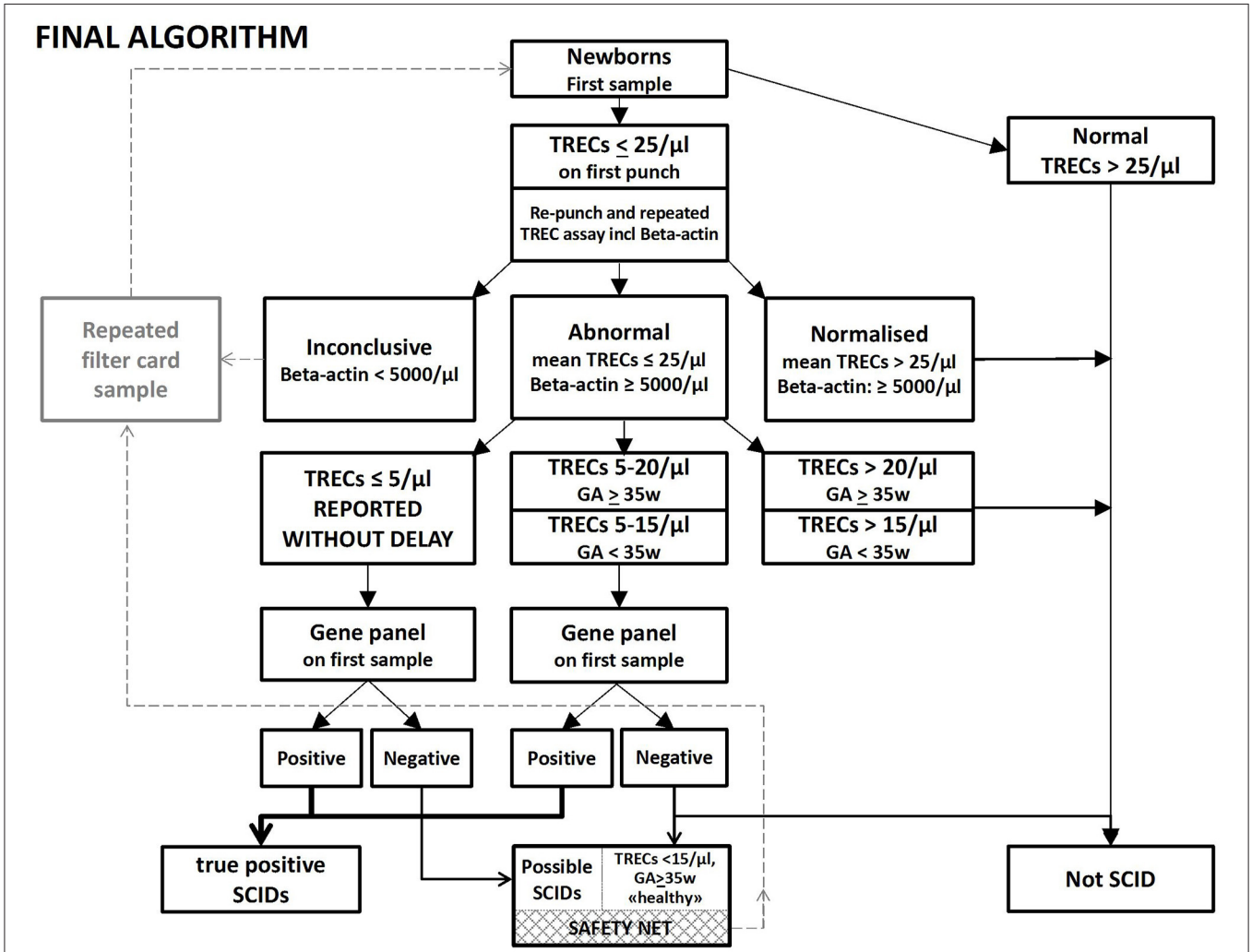

FIGURE 2 | Rapid NGS gene panel testing integrated in the NBS laboratory algorithm for SCID and other T cell lymphopenias, Final algorithm. Samples below 25 TRECs/μL are re-punched and TREC analyses repeated twice on DNA from the new punch. A new DBS sample is requested if low levels of β-actin (< 5,000/μL) are found. Samples with TRECs below 20/μL (15/μL for prematures) are immediately NGS gene panel tested. Samples with TRECs below 5/μL are reported without delay, regardless of the NGS findings. If TRECs are below 15/μL and NBS-NGS gene panel negative in an apparently healthy child with normal weight born to term, a second DBS sample is requested as a "safety net." And if the TREC values are low on the second sample as well, the pediatric immunologist will be alerted, and the child followed further up with clinical investigations.

and/or IgG positive, the CMV viral load in the child's urine and blood were measured after DNA extraction with MagNa Pure LC 96 (Roche Diagnostics, USA). An in-house method using a quantitative real-time PCR (LightCycler® 480, Roche Diagnostics, USA) with the conserved area of the polymerase gene UL54 as the target was used to quantify the CMV viral load. The detection limit was set to 36 IU/mL in each sample, and the quantification range to between 200 and 10,000,000 IU/mL. For comparison, the CMV viral load at birth was measured with the same qPCR method using DNA extracted from two new punches from the child's initial NBS filter card. This extraction method is described elsewhere (33).

RESULTS

We have explored the utility of rapid NGS integrated in the NBS laboratory algorithm for SCID. Out of the 21,232 samples tested in the prospective pilot project, three individuals were identified with SCID: on day 16, 13, and 11 after birth, respectively; one *IL2RG*, one *RAG2*, and one *RMPR* related SCID (**Table 1** and **Figure 1A**). Filter cards were received in the lab on day 4, 6, and 3 after birth, but the consent forms were sent separately, and since this was initially a research project and not part of the ordinary newborn screening, TREC testing was not performed until day 15, 12, and 11. The child with *RMRP*-SCID had complete Hirschsprung disease and died at 1 month of age. For the two others, within 4–5 days after TREC-testing and DNA extraction from the original NBS DBS sample, the sequencing results were available, utilizing NGS with Ion AmpliSeq^{TM} PID panel, and the molecular diagnosis was received at day 22 and day 17, respectively (**Table 1**). The *RAG2*-SCID patient had low intermediate TREC values, mean 9.7/μl, consistent with a leaky SCID. The molecular findings directed follow-up and therapy: the *IL2RG*-SCID underwent early hematopoietic stem cell transplantations (HSCT), without any complications; the leaky *RAG2*-SCID received prophylactic antibiotics, antiviral and antifungal treatment, immunoglobulin infusions, and was transplanted later, at 1 year of age (**Supplemental Table S1**). Both mothers were instructed (day 16 and day 17) to avoid breastfeeding their child for a short period of time before the maternal CMV status was determined by serological testing. One of the mothers was CMV positive (IgG positive, IgM negative, day 20) and breastfeeding was discontinued. The other mother was CMV negative (day 25) and breastfeeding was immediately re-started. Neither of these two babies became CMV infected prior to transplantation. Familial segregation testing confirmed the expected inheritance. The maternal uncle of the *IL2RG*-SCID patient had died of an infection in early childhood, and the maternal grandmother was a carrier of the *IL2RG* variant. Further genetic carrier testing has been offered for the potential carriers in the family. The parents of individual SCID_2 were confirmed carriers of the *RAG2* variant, and the SCID_3 individual's parents were both confirmed carriers of the *RMRP* variant (**Table 1**), hence, the apparent homozygous variants identified were located in trans alleles in these children, excluding allelic drop-out.

Only 37 (0.17%) of the 21,232 individuals in the pilot study had TRECs below 25/μl on the initial test (**Figure 1A**). Retesting was performed using both the original DNA-extract and DNA obtained from re-punching of the dried blood spot. Eight samples with TREC 0 on the first run had TREC-levels above 100/μl on the re-runs (data not shown) and were regarded as normal/negative. The 24 individuals in the pilot study with the lowest TREC values after retesting are presented in detail in **Supplemental Table S2**. Among these were nine premature babies with low birth weight, eight with intestinal malformations, and six with congenital heart disease either as a single feature or in combination with other comorbidities or prematurity. Altogether 21 of the 24 newborns with the lowest TRECs were hospitalized in neonatal intensive care units (NICU) (**Supplemental Table S2**), 20 of them at Oslo University Hospital (OUS). The PID panel was only run on selected patient samples. Genetic testing was not performed when there was another clear medical explanation for the low TREC such as intestinal malformation, multiple transfusions, extreme prematurity or intensive care after surgery (34). Out of the 21,232 samples TREC tested in the prospective pilot, nine underwent genetic testing as part of the screening (0.04%) (**Figure 1A**). Re-draws were requested in only 12 individuals (**Supplemental Table S2**). After the pilot, the cost of the total SCID screening reagents, including NGS of the selected cases with the lowest TREC values, was estimated to be 10 USD per sample.

The overall mean value in the pilot study was 285 TRECs/μl (5^{th}-95th centile range: 92.6–614 TRECs/μl), and median 244 TRECs/μl, when samples with values below 25/μl on the initial test were excluded. A significant correlation between TRECs and gestational age and birth weight was observed (**Supplemental Figure S1**). Premature babies and those with low birth weight had lower TREC values. The male newborns had slightly higher mean birth weight and lower TRECs values as compared to the girls (**Supplemental Figure S2**).

The Norwegian newborn screening unit is located at the hospital performing all HSCTs in children in Norway, and OUS is also the main referral hospital for critically ill neonates and infants. Six children, born during the period of the pilot study at hospitals not included in SCID screening, were referred to our clinic at 1–6 months of age based on symptoms such as infections and poor growth or dysmorphology with suspected immunodeficiency (**Table 2**). Their original newborn dried blood spots as well as a new sample were tested, and all had low TREC-levels and SCID-features at the time of referral. All except one had TRECs below the initial cut-off value (25/μl) at birth (**Supplemental Figure S2B**). A single patient (PID_6, **Table 2**) had TRECs of 72/μl at birth, but low TRECs (12/μl) when tested at 6 months. Gene panel testing on DBS DNA identified a *IKZF1*-related lymphoproliferative variant c.[476A>G];[=], p.Asn159Ser (35, 36). This variant occurred *de novo*. Directed by the genetic finding (35, 36), as the specific variant was reported to cause lymphoproliferative disorder, this child underwent HSCT, and is currently healthy 1 year after transplantation (37), demonstrating the utility of combining TREC testing and NGS as a diagnostic tool beyond newborn screening. However, in two of the other children (PID_1 and PID_2, **Supplemental Table S3**), both born small for gestational age (and named "SGA and leukopenia" in **Supplemental Figure S1B**), we were not able to identify the molecular cause of their disease. The last three had CHARGE syndrome, Down syndrome, and DiGeorge syndrome, respectively (PID_3, PID_4 and PID_5 in **Table 2** and **Supplemental Table S3**).

During the pilot study time period ∼130,000 children were born in Norway (https://www.ssb.no/fodte/; 56,633 year 2017, 58,890 year 2016, and 13,210 Oct-Dec 2015). The incidence of SCID (based on the *IL2RG*-, *RAG2*-, *RMPR*-, and the *CHD7*-SCID, plus the child with the bone marrow failure and yet unidentified DNA-repair disorder, PID_2 in **Table 2**) was 1:26,000.

With regard to the 2nd tier NGS results in the *retrospective* study we were able to confirm the following molecular findings: *IL2RG* hemizygous c.924+5G>A (NM_000206.2), *JAK3* compound heterozygous c.1837C>T;

c.1695C>A (NM_000215.3), *IL7R* homozygous c.707-2A>G (NM_002185.3), *LIG4* compound heterozygous c.1341G>T; c.482delC (NM_002312.3), *PGM3* homozygous c.737A>G (NM_015599.2), and the *RECQL4* heterozygous variant c.2269C>T (NM_004260.3), but not the intronic c.3056-3C>A which had been identified by WES and published earlier (22), since the predesigned gene panels had no amplicon which included this particular region of *RECQL4*. We were not initially able to detect any *ADA* sequence alteration(s) in the ADA-SCID sample. The patient had parents of Somali ethnicity, and by studying the raw BAM file it turned out that the PIDv1 gene panel was missing exon 1 where the Somali ADA founder mutation is located. Hence, PIDv2 was developed and used as a 2nd tier DNA assessment in the pilot project and the retrospective study (**Table 3** and **Supplemental Table S4**). DiGeorge syndrome deletion 22q11 was tested by *TBX1* dosage using bioinformatic interpretation of NGS data. Of note, 55% (five out of nine) of the known Ataxia-Telangiectasia patients would have been detected on initial screening using a TREC cut-off below 25 TRECs/μl (**Table 4**). As a result of our pilot project and retrospective studies, the Norwegian government and health authorities decided to mandate nationwide NBS for SCID.

PROOF-OF-PRINCIPLE FOR RAPID NGS IN NBS

When NBS for SCID was implemented nationwide in the ordinary screening panel, the customized targeted NBS-NGS core panel (See Methods and **Supplemental Table S7**) was used. The test algorithm and results of the first period of routine SCID screening are presented in **Figure 1B**. The first SCID patient was identified within 4 days after birth with zero TRECs/μl on 3 separate punches (Blood sample collected day 2, arrived at the central lab and the 1st TREC test performed day 3, two new punches and re-run TREC tests performed day 4, and β-actin tested in parallel). When the local hospital and the parents were informed about the SCID-suspicion, at day 4; the mother was immediately asked to discontinue breast-feeding. The mother was serological CMV tested, and at day 9 she turned out to be CMV positive (IgG pos/IgM neg, **Supplemental Table S5**), hence, breast-feeding was permanently avoided for this child. This story of the first SCID identified during routine screening (individual SCID_4, **Table 1**) demonstrates the applicability of rapid NGS integrated in the SCID screening lab testing algorithm: Using DNA from the original newborn screening dried blood spot sample, at day 8, it was clear that the male infant was homozygous for a *DCLRE1C* variant and had Artemis-SCID. The variant NM_001033855.2(DCLRE1C): c.[82C>G](;)[82C>G], p.Ala28Pro had previously been reported in T-B-NK+ SCID (38). A donor search was then initiated. While waiting for the transplantation, the infant was treated with prophylactic doses of antivirals, antifungals and antibiotics, plus immunoglobulin infusions. X rays and CT scans were avoided due to the molecular confirmation of radiosensitive SCID, and he followed a modified preconditioning HSCT protocol (**Table 1** and **Supplemental Table S5**).

Yet another baby born in 2018 (individual SCID_5, **Table 1** and **Supplemental Table S5**) was identified with SCID at day 13 with zero TRECs, and the *JAK3* variants detected by NBS-NGS core panel on day 15. His mother stopped breastfeeding on day 14 and was verified seropositive at day 17, but the baby had by then already acquired a CMV infection: CMV PCR was negative on the initial NBS filter card, while CMV in plasma was >1,000 IU/mL day 14. He then received antiviral treatment in line with recommendations by Vicetti Miguel et al. (3).

Furthermore, reactivation of CMV was observed 2 months post-HSCT with virus load increasing to >100,000 IU/mL. The reason for the increased turn-around-time and late screening in the JAK3 patient were delays both in the mail and the laboratory (Sample had been collected on day 2, but arrived in the NBS lab at day 8, and the TREC results were not ready until day 13, while the *JAK3* variants were identified day 15).

In the last SCID patient identified and reported here (individual SCID_7, **Table 1**), zero TRECs were detected in the filter card on day 4 after birth, molecular confirmation of *IL2RG* disease was ready on day 6, again demonstrating the value of rapid NGS in NBS. Breast-feeding was stopped immediately after the low TRECs were reported, the mother was tested and found CMV seropositive day 5, and the child was not CMV infected (SCID_7, **Supplemental Table S5**).

Out of the ∼88,000 newborns SCID screened during 2018 and 2019 (from Jan 1st 2018 to Aug 1st 2019) 81 had TRECs below 25/μl on the first test, 35 below 25/μl on the repeated test, and all of these 35 (0.4% of samples) were NGS tested (**Figure 1B**). Five had TRECs between zero and 5/μl and were immediately reported, and among them, one Nijmegen breakage syndrome (*NBN*) with SCID-like phenotype and three classical SCIDs (Artemis, *JAK3*, and *IL2RG*) were identified (**Table 1**). The last one with TRECs below 5/μl had Trisomy 21 was born prematurely with multiple anomalies (hydrops fetalis, congenital heart disease and arthrogryposis affecting all extremities), and later died at 1 month of age (CID_2 in **Table 1**, and in **Figure 1B**). Regarding the others ($n = 30$), none had definite diagnostic findings on the NBS gene panel testing. Twenty-one of the 30 infants were in NICU at the time of first sampling, and a repeat filter card blood test (redraw) was requested from 12 (received from 11) individuals; of which six had received total parenteral nutrition (TPN), and new samples were requested mainly due to the expectation of TPN interference in the biochemical screening markers for metabolic disorders on the first sample. Despite the lack of gene findings on our NBSv2 panel, redraws were specifically requested from five apparently healthy children born at term with low mean TREC values of 11.2, 11.4, 13.3, 17, and 23.4/μl, respectively (**Supplemental Table S6**). Four of them had normalized TREC values on the second sample, but one (individual CID_1 **Table 1**) had consistently low TRECs, no pathogenic gene findings on the NBSv2 or the PIDv2 panel, few T cells on flow cytometry and thymic aplasia on ultrasound. Further genetic testing with WES, chromosomal microarray and MLPA (multiplex ligation-dependent probe amplification) targeting single exons in *CHD7* did not reveal any genetic cause of the missing thymus and T cells (CID_1 in **Table 1** and **Supplemental Table S5**). One child with low TRECs and generalized skin disease, scaly erythroderma,

and therefore suspected Omenn syndrome had a homozygous variant of unknown significance (VUS) in *TTCA7*. The variant was evaluated to be a rare variant unrelated to disease, and whole exome sequencing (WES) later identified a homozygous deleterious variant in *SPINK5* causing Netherton syndrome. In another baby with mean TRECs of 9/μl and no findings on the NBS gene panel, WES later identified *PMM2*-CGD. None of these two babies were reported as SCID screening positives (**Supplemental Table S6**). No other children born within this time period (Jan 2018-Aug 2019) have yet been referred for SCID or severe T cell deficiency besides the ones identified through the national screening program. For the 3 SCID-patients and the Nijmegen breakage syndrome case with SCID-like phenotype identified by screening in the national program the TRECs results were ready at day 4, 13, 4, and 6, respectively, and their molecular diagnosis (*DCLRE1C, JAK3, IL2RG, NBN)* were molecularly confirmed on day 8, 15, 6, and 8 after birth, respectively, using the initial blood spot. Four SCIDs out of 88,000 tested reflects an incidence of 1 per 22,000 live births, 1: 29,300 without the NBN case.

DISCUSSION

To our knowledge we were the first country in Europe to provide nationwide SCID screening, although 10 years behind the first states in the US (39). Our nationwide SCID screening started 1 year after the Spanish region of Catalonia offered SCID screening to all newborns in their region (8). Catalonia has 7.5 million inhabitants, while Norway has 5.4 million inhabitants. The incidence of SCID in Catalonia was 1:130,000 births after 2 years (8), while we ascertained five times more cases: The incidence of SCID and severe T cell deficiencies was 1:26,000 in the pilot study and 1:22,000 after the screening was made nationwide, 1: 29,300 without the NBN case. A high incidence of SCID and severe T cell deficiencies has been reported in certain populations and ethnic groups (26, 40–42), as well as an increased incidence in the general population after the introduction of screening, contrasting with the pre-SCID-screening estimated incidence from historical data and medical records (43). With the introduction of NBS for SCID in the US, and based on more than 3 million screened babies, the overall incidence was revised to 1:58,000, which was nearly twice their pre-screening estimated numbers (44). In the US there are also population pockets with higher incidence of SCID due to founder mutations (42). The parents of the reported patients in our screening studies were a mixture of ethnic Norwegians and Norwegians with immigrant parents originating from Estonia, Lithuania, Poland, Turkey, and Somalia, which presently reflects the population in our country. Whether immigration has led to a changed incidence of SCID and T cell disorders in Norway remains to be documented, but certain disorders and founder variants such as in ADA-SCID (KID_4, **Table 3**) may have increased compared to our historical numbers (43). However, precautions need to be taken since our total numbers are small. Our numbers for other severe T cell disorders and atypical SCID are high, but should not be extrapolated to the whole population because of the small screening total and the short observation time. Several years of national screening is needed to provide a more robust estimate of the incidence of SCID and severe T cell disorders in our population. The true incidence of SCID in Norway is nevertheless probably much higher than the numbers estimated from the epidemiological study in Norway (1:100,000 live births) (43). It is unlikely that this is solely explained by the increase in immigrant child births. There is a high likelihood that several pre-screening SCID patients died of infections without receiving a SCID diagnosis (43).

Our national SCID screening program includes rapid and broad integrated molecular diagnostic testing on dried blood spots, and was the first to implement this routine worldwide (26). We have shown that the combination of TREC measurements and high throughput NGS analyses using targeted gene panels in newborn screening is an effective genotype-based approach to molecular diagnosis for affected infants. This combination also increases the diagnostic precision, limits false positives, and minimizes unnecessary contact with the families of healthy newborns; fulfilling a fundamental goal for newborn screening laboratories (45). Early identification of the molecular cause and disease mechanisms enable early protective interventions and targeted or curative therapy. In patients identified with radiosensitive SCID, precautions and modification regarding radiographic imaging and the use of DNA-damaging radiomimetic drugs in pre-treatment conditioning can immediately be addressed, such as in the Artemis-SCID (individual SCID_4, **Table 1** and **Supplemental Table S5**).

CMV can be a serious and fatal infection even in early identified, newborn-screened SCID infants (3, 4). Others have documented that more than 40% develop infections before HSCT (4) such as varicella (23), rubella, EBV, with CMV being the most common. Although patients diagnosed via NBS are less likely to have an infection before HSCT, avoiding post-natal CMV infection remains an important challenge (4, 46) The NBS algorithms that require a second dry blood spot have their final TREC results available only after 3–4 weeks of age (44), limiting the oppurtunities for early CMV intervention. A faster turn-around-time to a definite SCID diagnosis may be the most practical way to reduce CMV exposure time (45), and general viral infection rates (23). Six children had CMV seropositive mothers (one during the pilot and five from routine screening). Four of these individuals were identified early, within the first week of life, and did not acquire a CMV infection. Two infants were identified with SCID as late as 2 weeks after birth, and one of them developed a clinical CMV infection, the child with *JAK3*-SCID and complete absence of T cells. The other child had a leaky *RAG2*-SCID, and a virus protective effect of the residual T cells cannot be excluded. The CMV infection in the *JAK3*-SCID was presumably acquired postnatally, since CMV was not detected in the initial NBS filter card, but was found in plasma at 2 weeks of age. Not all congenital CMV infections are detected by CMV DNA analysis in DBS (47), but the increasing virus load observed in plasma over 2–3 weeks (individual SCID_5, **Table 1** and **Supplemental Table S5**), is in accordance with the known incubation time for CMV infection in infants (48).

And maternal transmission through breastfeeding is a plausible explanation. CMV is reactivated in nearly all latently infected mothers and is excreted in the breast milk (3, 48, 49). CMV can also be transmitted via saliva. If the SCID identification through screening proceeds seamlessly and without delay from sample collection to positive screening, and breastfeeding in seropositive mothers is prevented at an early stage, perhaps the most optimal pre-HSCT health condition can be achieved. However, this remains to be proven beyond the four individuals ascertained and reported here, since others have recently reported surprisingly few CMV infected among breast fed in their historical SCID data (50).

A critical step in the implementation of full scale screening was choosing the TREC cut-off and deciding how to handle samples which were positive after 1st tier analysis. With NGS integrated into the NBS algorithm, we chose a 1st tier TREC cut-off value of 25/μl. Other SCID screening algorithms, without NGS, regularly use lower cut-offs, request new samples and/or report more false positives (See paragraph below). Our relatively high TREC cut-off value allowed for identification of leaky SCID such as the *RAG2*-SCID in individual SCID_2 (**Table 1** and **Supplemental Table S1**). The majority of samples with moderately low TREC values (5–20/μL) showed no genetic variants associated with SCID or T cell deficiencies and were therefore not reported. Inevitably, a negative screening result cannot absolutely exclude all combined immunodeficiencies, exemplified by the patient with the *IKZF1* missense variant (PID_6, **Table 2**) who presented after the newborn period. Not all molecular aberrations are detected or known, shown by the two children born outside pilot region with leukopenia and small for gestational age (PID_1 and PID_2, **Table 2**), and the apparently healthy child with thymus aplasia born at term with normal birth weights (CID_1, **Table 1**). Novel genes related to disorders with congenital T cell deficiency are constantly being reported and consecutively included on the updated International Union of Immunological Societies, IUIS' lists of recognized human inborn errrors of immunity (20, 51). Thus, NGS cannot replace TRECs or other NBS markers, and a "safety net" is still needed for those with the lowest TRECs.

The heterogeneity of SCID requires a broad testing approach with large gene panels and ideally the ability to identify structural variants, such as large and small copy number variants (CNVs) (52). Among the few SCID and T cell deficient children we have NBS-NGS gene panel tested here, we have found various immunodeficiencies with multiple genetic causes (*IL2RG, RAG2, RMRP, IKZF1, CHD7, DCLRE1C, JAK3*), with different inheritance patterns (AR, AD, de novo, XL) and mutation types (i.e., CNVs in DiGeorge syndrome, intragenic *IKZF1* deletion and larger chromosomal aberrations such as Trisomy 21). The heterogeneity and the expanding number of immunodeficiency genes therefore require a broad list of genes and regular updates of the panel.

Based on the experiences from the prospective pilot study, the retrospective study and the first period of nationwide SCID screening, we propose the following refined and final algorithm (**Figure 2**): All samples below initial cut-off 25 TRECs/μL will be re-punched in duplicate. Samples with two new punches with mean TREC levels below 20 TRECs/μL (below 15/μL for premature babies, GA < 35 weeks) and normal levels of β-actin proceed to NGS gene panel testing. However, if two out of the three TREC measurements/analyses are below 5/μL, the pediatric immunologist and family should be called immediately, without awaiting the NGS results, breastfeeding stopped, maternal CMV serology checked and flow cytometry performed in the child. Samples with TREC values between 5 and 20/μL—predominantly from premature or NICU infants—where NGS does not reveal any disease-causing mutations, are signed out as normal/negative SCID screens. A "safety net" allows for the collection of a new dried blood spot sample for apparently healthy babies born at term with TRECs 5–15/μL, without pathogenic NGS findings, and with no explanation for the low TRECs such as transient disease, congenital heart defect or intestinal abnormality (**Figure 2**) (34, 53). These children are followed-up by the pediatric immunology team and further diagnostic and functional testing is performed. With this algorithm, classical SCID will be identified, the time from birth to definitive diagnosis substantially reduced, and the number of recalls and second samples minimized despite our high initial cut-off value. By strictly following this algorithm for the 88,000 screened newborn babies nationwide, all SCID and severe T cell deficiencies would have been identified and only three other redraws requested.

Other programs have reported 0.08–0.4% redraws and recalls (5, 8, 44, 54, 55), and 0.016–0.14% for lymphocyte flow cytometry and consultation (18, 44, 56), while our overall redraw rate was 0.07% (12 second filter card + 3 SCIDs) out of 21 000 in the pilot study and 0.02% (13 second filter card sample requested + 4 SCIDs) out of the 88,000 in the nationwide screening, We could have achieved recalls as low as 0.01% with only 3 "false positives" if we had strictly followed our final algorithm (**Figure 2**). Many of our second filter card requests were due to the interference of total parenteral nutrition (TPN) with the biochemical screening markers used in NBS for inborn errors of metabolism, not immunodeficiency (**Supplemental Table S6**).

With this approach we are also able to identify Ataxia Telangiectasia (AT), which was previously reported by Mallot et al. (57) and others (58). We have not identified any AT patient in the pilot, or so far in the nationwide NBS. Norway has a higher incidence of AT as compared to neighboring countries in Europe, and the Norwegian AT founder mutation is expected to have an impact on the number of AT patients identified by NBS in the long run (59, 60). A plan for the diagnostic process, follow-up and care of the family needs to be in place for the AT-group, where there is currently no curative treatment available for the neurological phenotype, and where the clinical signs of neurodegeneration with ataxia usually start at 1 year of age (61). HSCT has been controversial and avoided in this group of DNA repair disorders for many years, but may with modified preconditioning be safely used to cure the immunodeficiency (62). The child identified with Nijmegen breakage syndrome had zero TRECs and a SCID-like phenotype, and HSCT was considered, since it has been performed in other NBN cases (63), but not planned in this case. Others have also identified children with Nijmegen breakage syndrome by SCID screening (64).

The successful integration of NGS within NBS paves the way toward expanding the NBS program to further include other severe and treatable genetic disorders and immunodeficiencies such as severe congenital neutropenia, X-linked and autosomal recessive chronic granulomatous disease and congenital agammaglobulinemia. New screening methods for other PIDs have been developed, with or without DNA-based markers (11, 65–67), but some disorders require a genetic method as the 1st tier test. NBS in Norway is based on informed consent, and allows for *genetic* mass screening of selected disorders to be performed without formal genetic counseling. It has been very useful in cystic fibrosis screening (28), in screening for metabolic disorders with limited numbers of disease genes (68–71), and for SCID screening with multiple disease genes. Analysis of biobanked samples from patients with known PIDs to evaluate the test methods, were performed retrospectively. The pilot was performed prospectively for ethical reasons; we did not want to test for SCID retrospectively with the lost opportunity to intervene and treat the affected newborns. However, the administrative challenge of obtaining *written* research consents from 21,000 parents and manually linking this with the corresponding samples arriving for routine screening was laborious for the NBS laboratory and overwhelming for all the maternity wards involved. A more streamlined approach will be required for the evaluation of further expansions of our routine NBS program.

We have not performed a formal cost effectiveness calcultation as this was not part of the main scope of the study. The presented NGS dependent algorithm has shown itself to be economically practical in full production. As costs for sequencing fall, new methods which also allow the detection of deep intronic variants, CNVs and other structural variants will be applicable to DBS samples (72, 73). The ethical concerns with this approach need to be addressed. Extensive *in silico* filtering to only include the relevant genes will be needed. Obligations to look for findings in other disease related genes or to report carrier status must also be avoided in order to prevent the multiple ethical dilemmas which could otherwise arise.

The utility and efficacy of 2nd tier NGS in SCID screening has been documented. The use of the original screening DBS sample has the following advantages:

- Molecular results within 2–3 working days
- Reduced need for a second DBS sample
- Reduced the total number of recalls, redraws and false positives
- Reduced need for pediatric consultations and flow cytometry to exclude false positives among the lower intermediate TRECs (5–20/μl)
- No unnecessary worries inflicted on the parents to newborns with TRECs 5–20/μl
- Ability to detect atypical and leaky SCIDs and other T cell deficiencies
- Shorter time from birth to confirmation of the specific immunodeficiency disorder.

The disadvantages

- Equipment cost and running cost of NGS analyses
- Inability to identify immunodeficiencies with intermediate low TREC values and variants in genes not included in the current/available gene panels
- Structural variants and intronic variants can be missed by exon based targeted NGS panels.

In conclusion, NGS integrated in the nationwide NBS algorithm rapidly delineates the specific molecular diagnosis and provides useful information for management, targeted therapy, and follow-up. Rapid detection may prevent breast milk transmitted CMV infection and other pre-HSCT acquired infections, optimizing pre-HSCT health condition. Meanwhile, a higher TREC cut-off threshold combined with NGS allows the detection of atypical, leaky SCIDs and other combined immunodeficiencies. This study forms a proof-of-concept for the integration of NGS methods into NBS laboratory routines and lays the foundations for the use of NGS methods in rapid confirmatory testing for further NBS conditions. There is still development work to be done, not least with regard to continuously updating and evaluating the list of genes included in panels; but the combination of speed, precision, cost and generalisability are strong arguments for adopting of this approach. While the overall cost of NBS seen in isolation is increased, it is important to note that the efficiency gains, improved management, and increased precision enabled by this screening approach contribute to a significant saving from a broader healthcare perspective.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committees for Medical and Health Research Ethics South East Norway. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JS: designed the pilot, responsible for establishing all SCID screening laboratory activities including TREC test, and NGS analyses. KG: started the TREC testing and prior to the pilot. EL, MB, AT, LS, MY-A, SH, RH, and HG: TREC testing, NGS analyses and interpretation of the gene variants. HE and TØ: responsible for treatment of all the SCID patients in Norway, research partners. TA: former PI of the NBS for SCID project, initiated TREC project and pilot. LO: immunological laboratory investigations. GK: CMV testing. HS: WES tests and gene variant evaluation. LM: introduced the SCID screening to the clinic. ADR: bioinformatics and providing data sets. TT and JJ: responsible for the algorithm and medical follow-up in the

newborn screening. AL and AER: NICU patients. CA and JB: responsible for the bone marrow transplantations. TR: head of division, supported the pilot, involved in the project design and applied for nationwide SCID screening. RP: head of department, responsible for the newborn screening unit, and initiated the projects including nationwide SCID screening in Norway. AS-P: PI of the NBS for SCID project and the retrospective studies, designed the NBS gene panels and responsible for the gene variant evaluations. AS-P wrote the manuscript together with JS and KG. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01417/full#supplementary-material

Supplemental Figure S1 | (A) TREC values related to birth weight in all the 21,232 individuals included in the pilot study. **(B)** TREC values related to gestational age in all the 21,232 individuals included in the pilot study, plus the six individuals referred with severe primary immunodeficiencies, born in the same time period as the pilot, but at other hospitals.

Supplemental Figure S2 | (A) TREC values related to gender in 14,000 individuals (6,688 females and 7,250 males) included in the pilot study. **(B)** Birth weight related to gender in 14,000 individuals (6,688 females and 7,250 males) included in the pilot study.

Supplemental Table S1 | New SCIDs and severe T cell deficiencies detected in the pilot project.

Supplemental Table S2 | The individuals with the lowest TREC values in the pilot project.

Supplemental Table S3 | Individuals with PIDs born outside pilot test region.

Supplemental Table S4 | Retrospective study: TRECs and NGS on DNA from the original NBS DBS in individuals with known SCID or severe T cell deficiency.

Supplemental Table S5 | New SCIDs and severe PIDs identified during the national newborn screening.

Supplemental Table S6 | The individuals with the lowest TREC values on the national screening 2018-2019.

Supplemental Table S7 | Newborn screening gene panels, versions 1-4.

REFERENCES

1. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med.* (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
2. Dorsey MJ, Dvorak CC, Cowan MJ, Puck JM. Treatment of infants identified as having severe combined immunodeficiency by means of newborn screening. *J Allergy Clin Immunol.* (2017) 139:733–42. doi: 10.1016/j.jaci.2017.01.005
3. Vicetti Miguel CP, Mejias A, Ramilo O, Ardura MI, Sanchez PJ. Cytomegalovirus meningitis in an infant with severe combined immunodeficiency. *J Pediatr.* (2016) 173:235–7. doi: 10.1016/j.jpeds.2016.02.054
4. Heimall J, Logan BR, Cowan MJ, Notarangelo LD, Griffith LM, Puck JM, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. *Blood.* (2017) 130:2718–27. doi: 10.1182/blood-2017-05-781849
5. 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) 114:12–6. doi: 10.1016/j.jfma.2012.10.020
6. 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. *Int J Neonatal Screen.* (2017) 3:16. doi: 10.3390/ijns3030016
7. Somech R, Lev A, Simon AJ, Korn D, Garty BZ, Amariglio N, et al. Newborn screening for severe T and B cell immunodeficiency in Israel: a pilot study. *Isr Med Assoc J.* (2013) 15:404–9.
8. Argudo-Ramirez A, Martin-Nalda A, Marin-Soria JL, Lopez-Galera RM, Pajares-Garcia S, Gonzalez 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. doi: 10.3389/fimmu.2019.02406
9. Schütz C, Hauck F, Albert MH, Hönig M, Borte S, Wahn V, et al. Neugeborenenscreening auf schwere kombinierte Immundefekte. *Monatsschrift Kinderheilkunde.* (2019) 167:1027–32. doi: 10.1007/s00112-019-0743-z
10. Tagliaferri L, Kunz JB, Happich M, Esposito S, Bruckner T, Hubschmann D, et al. Newborn screening for severe combined immunodeficiency using a novel and simplified method to measure T-cell excision circles (TREC). *Clin Immunol.* (2017) 175:51–5. doi: 10.1016/j.clim.2016.11.016
11. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterstrom 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) 37:51–60. doi: 10.1007/s10875-016-0347-5
12. Gutierrez-Mateo C, Timonen A, Vaahtera K, Jaakkola M, Hougaard DM, Bybjerg-Grauholm J, et al. Development of a multiplex real-time PCR assay for the newborn screening of SCID, SMA, and XLA. *Int J Neonatal Screen.* (2019) 5:39. doi: 10.3390/ijns5040039
13. 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. *Int J f Neonatal Screen.* (2017) 3:27. doi: 10.3390/ijns3040027
14. Thomas C, Durand-Zaleski I, Frenkiel J, Mirallie S, Leger A, Cheillan D, et al. Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. *Clin Immunol.* (2019) 202:33–9. doi: 10.1016/j.clim.2019.03.012
15. 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. doi: 10.1016/j.clim.2017.05.007
16. Blom M, Bredius RGM, Weijman G, Dekkers EHBM, Kemper EA, Van den Akker-van Marle ME, et al. Introducing newborn screening for severe combined immunodeficiency (SCID) in the Dutch neonatal screening program. *Int J Neonatal Screen.* (2018) 4:40. doi: 10.3390/ijns4040040
17. Dorsey M, Puck J. Newborn screening for severe combined immunodeficiency in the US: current status and approach to management. *Int J Neonatal Screen.* (2017) 3:15. doi: 10.3390/ijns3020015
18. 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) 132:140–50. doi: 10.1016/j.jaci.2013.04.024
19. Kwan A, Puck JM. History and current status of newborn screening for severe combined immunodeficiency. *Semin Perinatol.* (2015) 39:194–205. doi: 10.1053/j.semperi.2015.03.004
20. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, 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) 40:24–64. doi: 10.1007/s10875-019-00737-x
21. Davies EG, Cheung M, Gilmour K, Maimaris J, Curry J, Furmanski A, et al. Thymus transplantation for complete DiGeorge syndrome:

European experience. *J Allergy Clin Immunol.* (2017) 140:1660–70.e1616. doi: 10.1016/j.jaci.2017.03.020
22. Stray-Pedersen A, Sorte HS, Samarakoon P, Gambin T, Chinn IK, Coban Akdemir ZH, et al. Primary immunodeficiency diseases: genomic approaches delineate heterogeneous Mendelian disorders. *J Allergy Clin Immunol.* (2017) 139:232–45. doi: 10.1016/j.jaci.2016.05.042
23. Yu H, Zhang VW, Stray-Pedersen A, Hanson IC, Forbes LR, de la Morena MT, et al. Rapid molecular diagnostics of severe primary immunodeficiency determined by using targeted next-generation sequencing. *J Allergy Clin Immunol.* (2016) 138:1142–51.e1142. doi: 10.1016/j.jaci.2016.05.035
24. Cirillo E, Cancrini C, Azzari C, Martino S, Martire B, Pession A, et al. Clinical, immunological, and molecular features of typical and atypical severe combined immunodeficiency: report of the italian primary immunodeficiency network. *Front Immunol.* (2019) 10:1908. doi: 10.3389/fimmu.2019.01908
25. 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. *J Allergy Clin Immunol.* (2014) 133:1092–8. doi: 10.1016/j.jaci.2013.09.044
26. 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. doi: 10.3389/fimmu.2018.00782
27. Kelly N, Makarem DC, Wasserstein MP. Screening of newborns for disorders with high benefit-risk ratios should be mandatory. *J Law Med Ethics.* (2016) 44:231–40. doi: 10.1177/1073110516654133
28. Lundman E, Gaup HJ, Bakkeheim E, Olafsdottir EJ, Rootwelt T, Storrosten OT, et al. Implementation of newborn screening for cystic fibrosis in Norway. Results from the first three years. *J Cyst Fibros.* (2016) 15:318–24. doi: 10.1016/j.jcf.2015.12.017
29. Heath EM, O'Brien DP, Banas R, Naylor EW, Dobrowolski S. Optimization of an automated DNA purification protocol for neonatal screening. *Arch Pathol Lab Med.* (1999) 123:1154–60.
30. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet.* (2000) 355:1875–81. doi: 10.1016/S0140-6736(00)02293-5
31. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform.* (2013) 14:178–92. doi: 10.1093/bib/bbs017
32. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* (2015) 17:405–24. doi: 10.1038/gim.2015.30
33. Barbi M, Binda S, Primache V, Caroppo S, Dido P, Guidotti P, et al. Cytomegalovirus DNA detection in Guthrie cards: a powerful tool for diagnosing congenital infection. *J Clin Virol.* (2000) 17:159–65. doi: 10.1016/S1386-6532(00)00089-5
34. Fullerton BS, Velazco CS, Hong CR, Carey AN, Jaksic T. High rates of positive severe combined immunodeficiency screening among newborns with severe intestinal failure. *JPEN J Parenter Enteral Nutr.* (2018) 42:239–46. doi: 10.1002/jpen.1013
35. Yoshida N, Sakaguchi H, Muramatsu H, Okuno Y, Song C, Dovat S, et al. Germline IKAROS mutation associated with primary immunodeficiency that progressed to T-cell acute lymphoblastic leukemia. *Leukemia.* (2017) 31:1221–3. doi: 10.1038/leu.2017.25
36. Boutboul D, Kuehn HS, Van de Wyngaert Z, Niemela JE, Callebaut I, Stoddard J, et al. Dominant-negative IKZF1 mutations cause a T, B, and myeloid cell combined immunodeficiency. *J Clin Invest.* (2018) 128:3071–87. doi: 10.1172/JCI98164
37. Kellner ES, Krupski C, Kuehn HS, Rosenzweig SD, Yoshida N, Kojima S, et al. Allogeneic hematopoietic stem cell transplant outcomes for patients with dominant negative IKZF1/IKAROS mutations. *J Allergy Clin Immunol.* (2019) 144:339–42. doi: 10.1016/j.jaci.2019.03.025
38. Pannicke U, Honig M, Schulze I, Rohr J, Heinz GA, Braun S, et al. The most frequent DCLRE1C (ARTEMIS) mutations are based on homologous recombination events. *Hum Mutat.* (2010) 31:197–207. doi: 10.1002/humu.21168
39. 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):88–95. doi: 10.1177/00333549101250S211
40. Suliaman F, Al-Ghonaium A, Harfi H. High incidence of severe combined immune deficiency in the eastern province of Saudi Arabia. *Pediatric Asthma Aller Immunol.* (2006) 19:14–8. doi: 10.1089/pai.2006.19.14
41. 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) 158:29–34. doi: 10.1016/j.clim.2015.02.015
42. Li L, Moshous D, Zhou Y, Wang J, Xie G, Salido E, et al. A founder mutation in Artemis, an SNM1-like protein, causes SCID in Athabascan-speaking Native Americans. *J Immunol.* (2002) 168:6323–9. doi: 10.4049/jimmunol.168.12.6323
43. Stray-Pedersen A, Abrahamsen TG, Froland SS. Primary immunodeficiency diseases in Norway. *J Clin Immunol.* (2000) 20:477–85. doi: 10.1023/A:1026416017763
44. 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) 312:729–38. doi: 10.1001/jama.2014.9132
45. 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) 143:51–63. doi: 10.1542/peds.2018-2300
46. van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal newborn screening for severe combined immunodeficiency (SCID). *Front Pediatr.* (2019) 7:373. doi: 10.3389/fped.2019.00373
47. Wang L, Xu X, Zhang H, Qian J, Zhu J. Dried blood spots PCR assays to screen congenital cytomegalovirus infection: a meta-analysis. *Virol J.* (2015) 12:60. doi: 10.1186/s12985-015-0281-9
48. Hamprecht K, Maschmann J, Vochem M, Dietz K, Speer CP, Jahn G. Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding. *Lancet.* (2001) 357:513–8. doi: 10.1016/S0140-6736(00)04043-5
49. Meier J, Lienicke U, Tschirch E, Kruger DH, Wauer RR, Prosch S. Human cytomegalovirus reactivation during lactation and mother-to-child transmission in preterm infants. *J Clin Microbiol.* (2005) 43:1318–24. doi: 10.1128/JCM.43.3.1318-1324.2005
50. Kelty WJ, Beatty SA, Wu S, Hanson IC, Demmler-Harrison GJ, Martinez CA, et al. The role of breast-feeding in cytomegalovirus transmission and hematopoietic stem cell transplant outcomes in infants with severe combined immunodeficiency. *J Allergy Clin Immunol Pract.* (2019) 7:2863–5.e2863. doi: 10.1016/j.jaip.2019.05.041
51. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, et al. International union of immunological societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *J Clin Immunol.* (2018) 38:96–128. doi: 10.1007/s10875-017-0464-9
52. Bayer DK, Martinez CA, Sorte HS, Forbes LR, Demmler-Harrison GJ, Hanson IC, et al. Vaccine-associated varicella and rubella infections in severe combined immunodeficiency with isolated CD4 lymphocytopenia and mutations in IL7R detected by tandem whole exome sequencing and chromosomal microarray. *Clin Exp Immunol.* (2014) 178:459–69. doi: 10.1111/cei.12421
53. Davey BT, Elder RW, Cloutier MM, Bennett N, Lee JH, Wang Z, et al. T-cell receptor excision circles in newborns with congenital heart disease. *J Pediatr.* (2019) 213:96–102. doi: 10.1016/j.jpeds.2019.05.061
54. Audrain MAP, Leger AJC, Hemont CAF, Mirallie 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). *J Clin Immunol.* (2018) 38:778–86. doi: 10.1007/s10875-018-0550-7
55. 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) 34:289–303. doi: 10.1007/s10875-014-0006-7

56. van der Spek J, Groenwold RH, van der Burg M, van Montfrans JM. TREC based newborn screening for severe combined immunodeficiency disease: a systematic review. *J Clin Immunol.* (2015) 35:416–30. doi: 10.1007/s10875-015-0152-6
57. Mallott J, Kwan A, Church J, Gonzalez-Espinosa D, Lorey F, Tang LF, et al. Newborn screening for SCID identifies patients with ataxia telangiectasia. *J Clin Immunol.* (2013) 33:540–9. doi: 10.1007/s10875-012-9846-1
58. Mandola AB, Reid B, Sirror R, Brager R, Dent P, Chakroborty P, et al. Ataxia telangiectasia diagnosed on newborn screening-case cohort of 5 years' experience. *Front Immunol.* (2019) 10:2940. doi: 10.3389/fimmu.2019.02940
59. Stray-Pedersen A, Jonsson T, Heiberg A, Lindman CR, Widing E, Aaberge IS, et al. The impact of an early truncating founder ATM mutation on immunoglobulins, specific antibodies and lymphocyte populations in ataxia-telangiectasia patients and their parents. *Clin Exp Immunol.* (2004) 137:179–86. doi: 10.1111/j.1365-2249.2004.02492.x
60. Laake K, Telatar M, Geitvik GA, Hansen RO, Heiberg A, Andresen AM, et al. Identical mutation in 55% of the ATM alleles in 11 Norwegian AT families: evidence for a founder effect. *Eur J Hum Genet.* (1998) 6:235–44. doi: 10.1038/sj.ejhg.5200181
61. Stray-Pedersen A, Borresen-Dale AL, Paus E, Lindman CR, Burgers T, Abrahamsen TG. Alpha fetoprotein is increasing with age in ataxia-telangiectasia. *Eur J Paediatr Neurol.* (2007) 11:375–80. doi: 10.1016/j.ejpn.2007.04.001
62. Bakhtiar S, Woelke S, Huenecke S, Kieslich M, Taylor AM, Schubert R, et al. Pre-emptive allogeneic hematopoietic stem cell transplantation in Ataxia Telangiectasia. *Front Immunol.* (2018) 9:2495. doi: 10.3389/fimmu.2018.02495
63. Deripapa E, Balashov D, Rodina Y, Laberko A, Myakova N, Davydova NV, et al. Prospective study of a cohort of Russian Nijmegen breakage syndrome patients demonstrating predictive value of low kappa-deleting recombination excision circle (KREC) numbers and beneficial effect of hematopoietic stem cell transplantation (HSCT). *Front Immunol.* (2017) 8:807. doi: 10.3389/fimmu.2017.00807
64. Patel JP, Puck JM, Srinivasan R, Brown C, Sunderam U, Kundu K, et al. Nijmegen breakage syndrome detected by newborn screening for T cell receptor excision circles (TRECs). *J Clin Immunol.* (2015) 35:227–33. doi: 10.1007/s10875-015-0136-6
65. Baron U, Werner J, Schildknecht K, Schulze JJ, Mulu A, Liebert UG, et al. Epigenetic immune cell counting in human blood samples for immunodiagnostics. *Sci Transl Med.* (2018) 10:eaan350. doi: 10.1126/scitranslmed.aan3508
66. Borte S, Fasth A, von Dobeln U, Winiarski J, Hammarstrom L. Newborn screening for severe T and B cell lymphopenia identifies a fraction of patients with Wiskott-Aldrich syndrome. *Clin Immunol.* (2014) 155:74–8. doi: 10.1016/j.clim.2014.09.003
67. Collins CJ, Chang IJ, Jung S, Dayuha R, Whiteaker JR, Segundo GRS, et al. Rapid multiplexed proteomic screening for primary immunodeficiency disorders from dried blood spots. *Front Immunol.* (2018) 9:2756. doi: 10.3389/fimmu.2018.02756
68. Smon A, Repic Lampret B, Groselj U, Zerjav Tansek M, Kovac J, Perko D, et al. Next generation sequencing as a follow-up test in an expanded newborn screening programme. *Clin Biochem.* (2018) 52:48–55. doi: 10.1016/j.clinbiochem.2017.10.016
69. Boemer F, Fasquelle C, d'Otreppe S, Josse C, Dideberg V, Segers K, et al. A next-generation newborn screening pilot study: NGS on dried blood spots detects causal mutations in patients with inherited metabolic diseases. *Sci Rep.* (2017) 7:17641. doi: 10.1038/s41598-017-18038-x
70. Luo X, Wang R, Fan Y, Gu X, Yu Y. Next-generation sequencing as a second-tier diagnostic test for newborn screening. *J Pediatr Endocrinol Metab.* (2018) 31:927–31. doi: 10.1515/jpem-2018-0088
71. Yang Y, Wang L, Wang B, Liu S, Yu B, Wang T. Application of next-generation sequencing following tandem mass spectrometry to expand newborn screening for inborn errors of metabolism: a multicenter study. *Front Genet.* (2019) 10:86. doi: 10.3389/fgene.2019.00086
72. Berg JS, Agrawal PB, Bailey DB Jr, Beggs AH, Brenner SE, Brower AM, et al. Newborn sequencing in genomic medicine and public health. *Pediatrics.* (2017) 139:e20162252. doi: 10.1542/peds.2016-2252
73. Bassaganyas L, Freedman G, Vaka D, Wan E, Lao R, Chen F, et al. Whole exome and whole genome sequencing with dried blood spot DNA without whole genome amplification. *Hum Mutat.* (2018) 39:167–71. doi: 10.1002/humu.23356

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Establishing Simultaneous T Cell Receptor Excision Circles (TREC) and K-Deleting Recombination Excision Circles (KREC) Quantification Assays and Laboratory Reference Intervals in Healthy Individuals of Different Age Groups in Hong Kong

Janette S. Y. Kwok[1], Stephen K. F. Cheung[1], Jenny C. Y. Ho[1], Ivan W. H. Tang[1], Patrick W. K. Chu[1], Eric Y. S. Leung[1], Pamela P. W. Lee[2], Daniel K. L. Cheuk[2], Vincent Lee[3], Patrick Ip[2] and Y. L. Lau[2]*

[1] Division of Transplantation and Immunogenetics, Department of Pathology, Queen Mary Hospital, Hong Kong, Hong Kong, [2] Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Hong Kong, Hong Kong, [3] Department of Paediatrics, The Chinese University of Hong Kong, Hong Kong, Hong Kong

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***Correspondence:**
Janette S. Y. Kwok
kwoksy@ha.org.hk

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The clinical experience gathered throughout the years has raised awareness of primary immunodeficiency diseases (PIDD). T cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) assays for thymic and bone marrow outputs measurement have been widely implemented in newborn screening (NBS) programs for Severe Combined Immunodeficiency. The potential applications of combined TREC and KREC assay in PIDD diagnosis and immune reconstitution monitoring in non-neonatal patients have been suggested. Given that ethnicity, gender, and age can contribute to variations in immunity, defining the reference intervals of TREC and KREC levels in the local population is crucial for setting up cut-offs for PIDD diagnosis. In this retrospective study, 479 healthy Chinese sibling donors (240 males and 239 females; age range: 1 month−74 years) from Hong Kong were tested for TREC and KREC levels using a simultaneous quantitative real-time PCR assay. Age-specific 5^{th}–95^{th} percentile reference intervals of TREC and KREC levels (expressed in copies per μL blood and copies per 10^6 cells) were established in both pediatric and adult age groups. Significant inverse correlations between age and both TREC and KREC levels were observed in the pediatric age group. A significant higher KREC level was observed in females than males after 9–12 years of age but not for TREC. Low TREC or KREC levels were detected in patients diagnosed with mild or severe PIDD. This assay with the established local reference intervals would allow accurate diagnosis of PIDD, and potentially monitoring immune reconstitution following haematopoietic stem cell transplantation or highly active anti-retroviral therapy in the future.

Keywords: T cell receptor excision circles, K-deleting recombination excision circles, primary immunodeficiency, immune reconstitution, reference interval

INTRODUCTION

T and B cells undergo V(D)J recombination to generate diverse and functional TCR and BCR repertoires, and these are crucial processes in the maturation of T and B cells that allow the recognition of unlimited numbers of antigens (1). During the TCR rearrangement process, excised DNA fragments create T cell receptor excision circles (TREC) that are exported to the T cell cytoplasm (2, 3). In particular, the δRec-ψJα signal joint TREC (sjTREC) is produced during TCRD deletion and detected in ∼70% of (alpha-beta) αβ T cells. They are considered the most optimal target to measure the evaluation of thymic output (4–6). Kappa-deleting recombination excision circles (KREC) are produced during BCR rearrangement in naïve B cells and are analogous to TREC (7). Similarly, sjKREC formed during intronRSS-Kde rearrangements in IGK locus is a robust target for the evaluation of B cell neogenesis from bone marrow (8, 9).

Both TREC and KREC are stable and non-replicative, and are subsequently diluted during cell proliferation (10, 11). Hence, TREC and KREC analyses have been widely applied in different clinical settings to evaluate thymic and bone marrow output. Measurement of TREC and KREC levels in peripheral blood can be used for screening of Primary immunodeficiency diseases (PIDD). PIDD, also known as inborn errors of immunity (IEI), are a group of disorders that lead to defects in the development or function of the immune system. The international Union of Immunological Societies (IUIS) has classified and described over 400 PIDD (12). There is an increasing number of PIDD due to an updated definition and advancements in diagnostic technology. The disease prevalence is reported to be as high as 127 in 100,000 (13, 14), and higher rates are expected in regions where consanguinity is more common. In Hong Kong, as there is no formal registry for PIDD, the Asian Primary Immunodeficiency Network (APIN) was formed to collect data on PIDD with a mission to improve care, education, and research (15, 16). Up to January 2020, over 140 local and 750 overseas PIDD patients referred via APIN have been diagnosed at the Department of Pediatrics and Adolescent Medicine, Queen Mary Hospital, the University of Hong Kong. Pediatric patients with PIDD are more likely to have recurrent bacterial or fungal infections (17). Severe forms of PIDD such as Severe Combined Immunodeficiency (SCID) are highly fatal if diagnosis and treatment are delayed, particularly after the onset of such infections.

Since 2015, newborn screening (NBS) using TREC levels for the early identification of SCID has been implemented in Israel, New Zealand, Norway, Taiwan, Switzerland, Germany, Iceland, Italy and several regions in Canada, United States, and Australia (18–20). Recently, Sweden, Spain, and Saudi Arabia have evaluated the application of both TREC and KREC levels for screening of SCID and agammaglobulinemia (6, 21, 22). The reference intervals and cut-off values TREC and KREC have been established for neonates. Studies have demonstrated the impact of aging on thymopoiesis and bone marrow output (23, 24). In order to interpret the TREC and KREC levels for non-neonatal PIDD patients, it is necessary to establish local and ethnic reference intervals in healthy individuals of different age groups.

In this study, we measured the TREC and KREC levels of healthy Chinese individuals in Hong Kong with essential age groups of 0–18 years, as PIDD occurs in patients mainly in this age range (16, 25). We also determined the reference intervals for adults in age groups of 19–74 years for applications in thymic and/or bone marrow output monitoring for post-HSCT patients. The effects of age and gender on the KREC and TREC levels were also analyzed. The analysis of TREC and KREC levels was performed using a multiplex real-time PCR method and the values were expressed in both copies per μL blood and copies per 10^6 cells.

MATERIALS AND METHODS

Subjects

Archived DNA extracted from whole blood specimens from 479 healthy Chinese sibling donors aged 1 month−74 years collected during 2011–2019 for work-up for related patients requiring HSCT were used in this study. All healthy controls underwent thorough clinical evaluations and were screened for a normal blood cell count. Blood samples from 12 PIDD patients with definitive diagnosis of SCID ($n = 2$), X-SCID ($n = 3$), Agammaglobulinemia ($n = 3$), DiGeroge Syndrome (DGS, $n = 1$), and Activated PI3K-Delta Syndrome (APDS, $n = 1$), GATA2 deficiency ($n = 1$) and X-linked hyper-IgM syndrome (HIGM, $n = 1$) were used as disease controls. Reference DBS specimens were generous gifts from Dr. Francis Lee, Centers for Disease Control and Prevention (CDC), US. This study was carried out in accordance with the Declaration of Helsinki and the ICH-GCP. The protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKWC IRB No. UW 18-185). The tests were performed at the Division of Transplantation and Immunogenetics, Queen Mary Hospital, Hong Kong.

DNA Extraction From Whole Blood Specimens

Genomic DNA from EDTA whole blood samples was extracted by a magnetic beads-based purification method using the TBG EZbead blood DNA Extraction Kit (Texas BioGene Inc., Taiwan) according to the manufacturer's instructions. DNA extracted from whole blood samples (300 μL) was eluted in 100 μL Tris-EDTA buffer and stored at −70°C. DNA purity was assessed using spectrophotometer and all samples had OD260/280 ratio between 1.7 and 2.0. DNA integrity was assessed by loading 50 ng DNA in 1% agarose gel electrophoresis and no DNA degradation was detected.

DNA Elution From Dried Blood Spot Specimens

Dried blood spot (DBS) cards were stored in low-gas permeable bags at −20°C according to Clinical and Laboratory Standards Institute (CLSI) guideline NBS06-A. DBS discs (2 mm) were punched and washed once in 100 μL Qiagen DNA elution buffer with shaking at 1,500 rpm for 10 min. The wash buffer was removed and a fresh 40 μL DNA elution buffer was added and

TABLE 1 | Primer and probe sequences of TREC, KREC, and β-actin.

		Name	Sequence (5′->3′)
TREC	Forward	TREC-Forward	CACATCCCTTTCAACCATGCT
	Reverse	TREC-Reverse	GCCAGCTGCAGGGTTTAGG
	Probe	TREC-FAM	ACACCTCTGGTTTTTGTAAAGGTGCCCACT
KREC	Forward	KREC-Forward	TCCCTTAGTGGCATTATTTGTATCACT
	Reverse	KREC-Reverse	AGGAGCCAGCTCTTACCCTAGAGT
	Probe	KREC-HEX	TCTGCACGGGCAGCAGGTTGG
β-actin	Forward	ACTB-Forward	ATTTCCCTCTCAGGCATGGA
	Reverse	ACTB-Reverse	CGTCACACTTCATGATGGAGTTG
	Probe	ACTB-Cy5	GTGGCATCCACGAAACTA

TABLE 2 | Number of subjects in different age groups.

Age (years)	Male (*n*)	Female (*n*)	Total
<1	5	4	9
1–4	32	25	57
5–8	37	30	67
9–12	25	34	59
13–18	51	51	102
19–30	20	18	38
31–40	19	24	43
41–50	16	15	31
51–60	16	15	31
>61	19	24	43
Total	240 (50%)	239 (50%)	479

subsequently heated at 95°C for 30 min. The eluted DNA in the supernatant was collected for TREC/KREC analysis and the TREC results were compared with those measured using CDC in-house assay. TREC/KREC analysis of 3 different spots on the same DBS sample were performed.

Quantitative PCR Assay

Levels of TREC, KREC, and β-actin (internal control) were simultaneously quantified in a 20-μL reaction volume containing 5 μL DNA, 4 μL LightCycler Multiplex DNA Master Mix (Roche Diagnostics, Germany), 500 nM primers (TREC primer, KREC primer, and β-actin primer), and 125 nM probes (FAM-labeled TREC probe, HEX-labeled KREC probe, and Cy5-labeled β-actin probe; Integrated DNA Technologies, Singapore). The primer and probe sequences are listed in **Table 1**, and their designs have been previously described by Chan et al. and Sottini et al. (18, 26). The PCR analysis was performed using the LC480 II Real-Time PCR (RT-PCR) System (Roche Diagnostics, Germany) with PCR conditions of 5 min at 95°C followed by 45 cycles of 5 s at 95°C and 1 min at 60°C. The TREC/KREC plasmids were a generous gift from Dr. Sottini (26). The β-actin plasmid coding the human β-actin DNA sequence was commercially manufactured (Integrated DNA Technologies, Singapore). Standard curves for the quantification of TREC, KREC, and β-actin were obtained by using 10-fold serially diluted TREC, KREC, and β-actin plasmids (1×10^6-1×10 copies/reaction). The copies of TREC and KREC were calculated and expressed as copies/μL blood or copies/10^6 nucleated cells as follow:

$$\text{TREC or KREC copies}/\mu\text{L blood} = \frac{Copy\ number\ of\ TREC\ or\ KREC\ x\ Eluted\ volume}{Blood\ volume\ for\ DNA\ extraction\ x\ 5\ uL}$$

$$\text{TREC or KREC copies}/10^6\ \text{cells} = \frac{Copy\ number\ of\ TREC\ or\ KREC}{(Copy\ number\ of\ \beta - actin/2)} \times 10^6$$

Statistical Analysis

Analysis of the data was performed using the Prism program version 5.01. Data were expressed as median ± SD or range and 5^{th}–95^{th} percentile for quantitative non-parametric measures, and both number and percentage for categorized data. Mann-Whitney *U*-test was used for comparisons between two independent groups for non-parametric data. Spearman's correlation coefficient test was performed to assess the strength of the relationship between studied parameters and age. A *p*-value of less than 0.05 was considered significant.

RESULTS

This study included 294 healthy pediatric sibling donors (150 males and 144 females; age range: 1 month−18 years; median age: 9.9 years) and 185 adult donors (90 males and 95 females; median age: 44.1 years). The number of males and females in the different age groups are listed in **Table 2**. The TREC and KREC levels were calculated as copies/μL blood and copies/10^6 cells. The medians and ranges of TREC and KREC levels of male and female in different age groups were provided in the **Supplementary Tables 1**, **2**. Samples with TREC and KREC levels below the detection limit (10 copies/reaction) were checked for the presence of β-actin copies to confirm that the low levels were not due to amplification errors and therefore amplification failure can be ruled out.

TREC and KREC Levels Declined With Increasing Age

The TREC and KREC levels were plotted against age of healthy individuals to assess any overall correlations (**Figures 1**, **2**). A distinct pattern was observed in pediatric individuals (0–18 years) compared to adults (>18 years), thus the data was divided into pediatric and adult age groups and analyzed separately. The highest levels of TREC and KREC were observed in those of early age. There was a significant inverse correlation between TREC levels (both copies/μL blood and copies/10^6

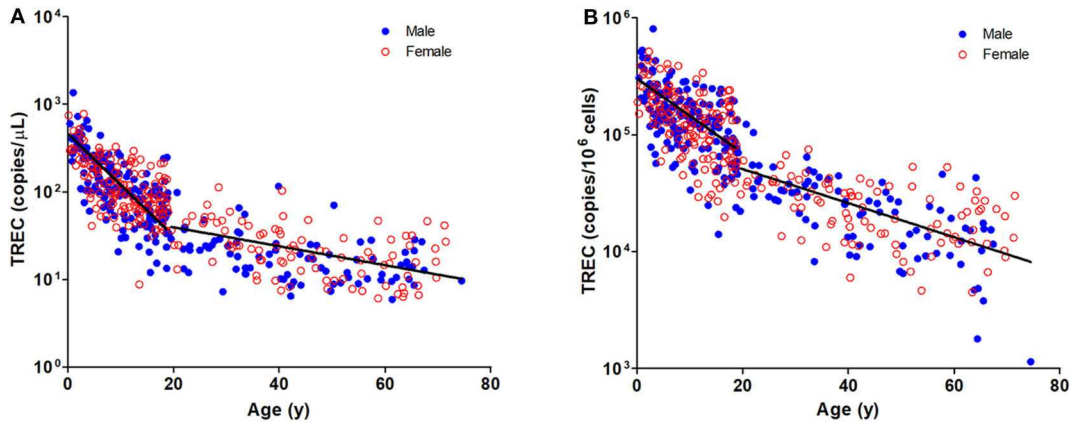

FIGURE 1 | Dot Plot showing TREC copies/μL blood **(A)** and TREC copies/10^6 cells **(B)** among the study age groups. Blue full circles represent healthy males and red empty circles represent healthy females. A significant inverse correlation was observed between TREC levels and both pediatric and adult age groups (Pediatric: $r = -0.6488$, $p < 0.0001$ for copies/μL and $r = -0.5487$, $p < 0.0001$ for copies/10^6 cells; Adult: $r = -0.4924$, $p < 0.0001$ for copies/μL and $r = -0.6289$, $p < 0.0001$ for copies/10^6 cells).

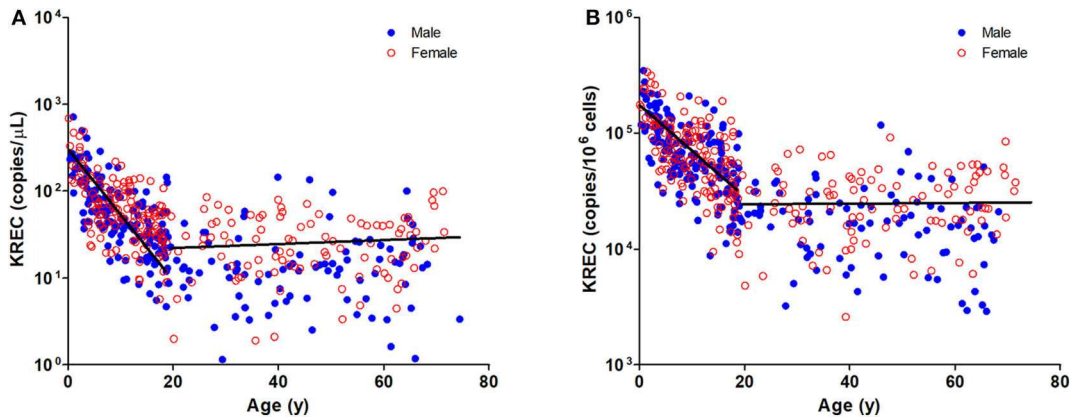

FIGURE 2 | Dot Plot showing KREC copies/μL blood **(A)** and TREC copies/10^6 cells **(B)** among the study age groups. Blue full circles represent healthy males and red empty circles represent healthy females. A significant inverse correlation was observed between KREC levels and pediatric age groups (Pediatric: $r = -0.6577$, $p < 0.0001$ for copies/μL and $r = -0.6241$, $p < 0.0001$ for copies/10^6 cells; Adult: $r = 0.0903$, $p = 0.2216$ for copies/μL and $r = -0.0171$, $p = 0.8176$ for copies/10^6 cells).

cells) and age for both pediatric and adult groups. The rate of decline in TREC level with age was greater in the pediatric group and slowed down with age in the adult group. A significant inverse correlation between KREC level (both copies/μL blood and copies/10^6 cells) was also found in the pediatric group, but not in the adult group. The KREC level varied but was maintained at a stably low level in adult. No difference in TREC level was observed between males and females in all age groups. A significantly higher KREC level (copies/μL blood) was detected in females than in males after 9–12 years of age (**Figure 3**). In addition, a significant positive correlation was observed between units in copies/μL blood and units in copies/10^6 cells for both TREC and KREC levels (**Figure 4**).

Reference Intervals of Healthy Individuals for Different Age Group

To establish reference intervals for TREC and KREC levels in the Hong Kong Chinese population, subjects were divided into 10 different age groups (5 groups for pediatric age and 5 groups for adults) and reference intervals were expressed as median and 5^{th}–95^{th} percentile range (**Table 3**). The lower threshold (5^{th} percentile) of reference ranges for TREC (copies/μL blood and copies/10^6 cells) in pediatric age groups were 223 and 151,107 (<1 year), 74 and 66,845 (1–4 years), 53 and 58,281 (5–8 years), 30 and 38,206 (9–12 years), 21 and 27,173 (13–18 year); and in adult groups were 12 and 20,831 (19–30 years), 10 and 8,436 (31–40 years), 7 and 6,644 (41–50 years), 0 and 0 (51–60 year), and 0 and 0 (>61 years). The lower threshold (5^{th} percentile)

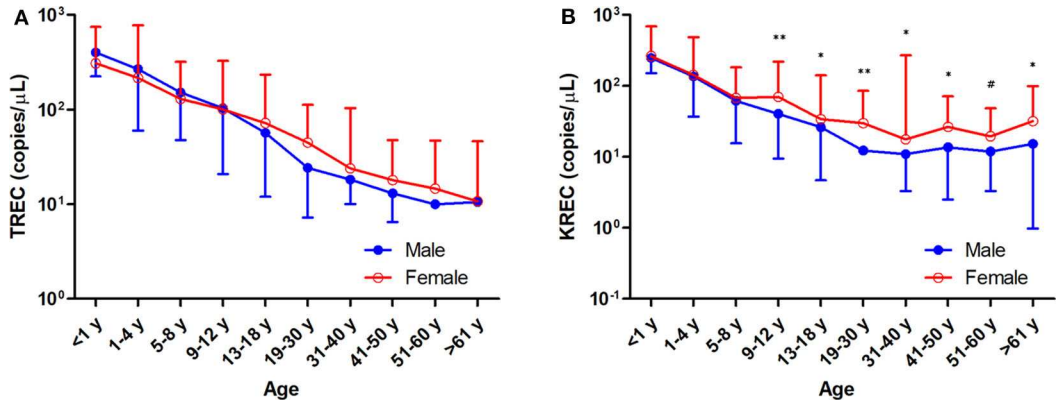

FIGURE 3 | Trend of TREC copies/μL blood **(A)** and KREC copies/μL blood **(B)** among the study age groups. Data is expressed as median ± range. Blue full circles represent healthy males and red empty circles represent healthy females. A significant higher KREC level was observed for age groups after 9–12 years, except for 51–60 years. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p = 0.0510$.

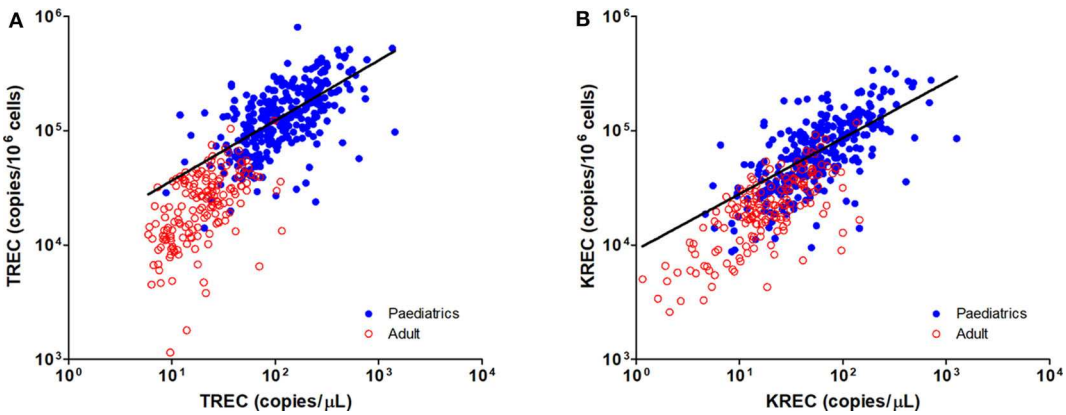

FIGURE 4 | Dot Plot showing the correlations between copies/μL blood and copies/10^6 cells for TREC **(A)** and KREC **(B)** levels. Blue full circles represent healthy pediatric individuals and red empty circles represent healthy adults. A significant positive correlation was observed between both units for TREC and KREC levels (TREC level: r = 0.8319, $p < 0.0001$; KREC level: r = 0.7794, $p < 0.0001$).

of reference ranges for KREC (copies/μL blood and copies/10^6 cells) in pediatric age groups were 134 and 115,946 (<1 year), 31 and 35,491 (1–4 years), 21 and 29,471 (5–8 years), 16 and 25,425 (9–12 years), 8 and 11,684 (13–18 years); and in adult groups were 1 and 3,063 (19–30 years), 2 and 6,098 (31–40 years), 4 and 7,363 (41–50 years), 3 and 5,566 (51–60 years) and 1 and 2,907 (>61 years).

Validation of Assay With Reference DBS Specimens

DNA from the 21 reference DBS specimens were eluted and tested using the TREC and KREC assays (**Table 4**). "Expected" TREC levels of the reference samples measured using an in-house RT-PCR TREC assay were provided by Dr. Francis Lee, CDC, US. Beta-actin (internal control) was detected in all DBS specimens except the negative control that was prepared from leukocyte-depleted blood and served as a negative control. Out of the 15 DBS specimens prepared from normal cord blood (within TREC reference range), we detected positive TREC levels ranging from 144 to 514 copies/μL blood. For the five SCID-like DBS samples prepared from PBMC-depleted blood, TREC levels were all below the detection limit. These results were in concordance with the expected results, and the TREC levels measured using our assay were highly correlated with those measured by the CDC (**Supplementary Figure 1**). On the other hand, there was no reference KREC samples available, no comparison could be done.

Use of TREC and KREC Assays in PIDD Patients

Blood samples from patients diagnosed with PIDD were tested with the TREC and KREC assays and compared with their age-matched reference intervals. The results and genetic diagnosis of the patients are listed in **Table 5**. Patient P1 presented with recurrent infections as well as low CD4+ T cell and CD19+ B cell counts. The patient was diagnosed with compound heterozygous mutations in RAG1 and both TREC and KREC levels were

TABLE 3 | Reference intervals of TREC and KREC in different age groups.

Age group (years)	*n*	TREC (copies/μL)		TREC (copies/10^6 cells)		KREC (copies/μL)		KREC (copies/10^6 cells)	
		Median ± SD	5^{th}–95^{th} percentile	Median ± SD	5^{th}–95^{th} percentile	Median ± SD	5^{th}–95^{th} percentile	Median ± SD	5^{th}–95^{th} percentile
<1	9	313 ± 363	223–1,355	304,896 ± 136,873	151,107–526,408	249 ± 219	134–713	217,210 ± 74,795	115,946–345,920
1–4	57	249 ± 170	74–656	213,155 ± 134,031	66,845–461,833	140 ± 116	31–470	114,289 ± 69,069	35,491–275,571
5–8	67	144 ± 83	53–284	151,838 ± 84,272	58,281–357,873	65 ± 47	21–171	69,186 ± 35,861	29,471–150,023
9–12	59	101 ± 73	30–279	125,197 ± 68,390	38,206–285,532	51 ± 46	16–186	64,424 ± 40,532	25,425–182,274
13–18	102	64 ± 50	21–209	86,770 ± 58,610	27,173–229,987	30 ± 28	8–107	36,610 ± 33,621	11,684–116,963
19–30	38	29 ± 22	12–99	38,543 ± 20,899	20,831–104,674	19 ± 20	1–70	21,262 ± 15,840	3,063–66,698
31–40	43	22 ± 23	10–96	29,356 ± 15,133	8,436–61,274	14 ± 28	2–77	18,056 ± 14,683	6,098–59,155
41–50	31	17 ± 13	7–57	20,996 ± 10,369	6,644–40,467	16 ± 29	4–114	22,835 ± 23,488	7,363–103,165
51–60	31	11 ± 10	0–37	12,706 ± 14,525	0–52,945	13 ± 13	3–45	17,933 ± 15,506	5,566–58,917
>61	43	11 ± 12	0–39	11,668 ± 11,130	0–35,331	22 ± 26	1–99	21,092 ± 18,671	2,907–53,655

TABLE 4 | Comparison of TREC results with reference DBS specimens.

		Reference results			Total
		Normal (TREC +ve)	SCID-like (TREC –ve)	Unsatisfactory sample (reference gene out of range)	
Measured results	TREC +ve ACTB +ve	15	0	0	**15**
	TREC –ve ACTB +ve	0	5	0	**5**
	TREC –ve ACTB –ve	0	0	1	**1**
	Total	**15**	**5**	**1**	**21**

very low or undetectable. Patient P2 has a history of severe chest infection, profound T cell lymphopenia and borderline low B cell count. The patient was diagnosed with compound heterozygous mutations in IL7RA and Diffuse Large B cell Lymphoma (DLBCL). The patient had much lower TREC and KREC levels than the age-matched reference intervals. Patients P3, P4, and P5 were classical $T^-B^+NK^+$ X-linked SCID patients with IL2RG defects. They all had undetectable TREC which was in agreement with their low T cell counts. Patient P5 had lower KREC level which could be explained by the low B cell count. Patient P6 was diagnosed with partial DGS, and had low TREC and normal KREC levels compared with the corresponding age group. Patient P7 was diagnosed with APDS, and had both lower TREC and KREC levels compared with the age-matched reference interval which were in concordance with the low T and B cells counts. Patients P8 and P9 were both diagnosed with XLA and had defects in BTK gene. Patient P10 had agammaglobulinemia phenotype with unknown genetic cause as found by Whole Exome Sequencing while patient P11 was diagnosed with GATA2 deficiency. Patients P8, P9, P10, and P11 were T^+B^- PIDD and they all had normal TREC but very low KREC levels. Patient P12 was diagnosed with HIGM and the TREC and KREC levels were normal which were consistent with normal T and B cell counts. The 5^{th} percentile lower thresholds of TREC and KREC were able to detect severe and mild abnormalities in the thymic function and B cell neogenesis in these patients.

DISCUSSION

To the best of our knowledge, this is the first and largest study to determine TREC and KREC reference levels in healthy Chinese pediatric and adult individuals. A total of 479 healthy individuals were divided into 10 different age groups for calculating the age-specific reference intervals, with the lower threshold defined as the 5^{th} percentile. The pediatrics age ranges were selected with reference to previous studies (26, 27). We also determined the reference intervals for adults in age groups between 19 and 74 years for the potential applications in thymic and/or bone marrow output monitoring for post-HSCT patients and HIV patients.

We and other groups have observed a significant inverse correlation of TREC levels with increasing age (28–31). A greater downward trend was observed in the pediatric group than in adults, which is in agreement with other studies (28, 32). We detected the highest TREC and KREC levels in infants <1 year old (33, 34), which may be explained by the continual thymic development in the first year of life (35, 36). We found the TREC levels decreased with age, which is suggested to be related to thymic involution (37), and lower TREC and KREC levels result from dilution during homeostatic replication of T or B memory cells or antigen-induced proliferation (9, 38). In contrast to the steady decline of TREC levels in the adult age group, KREC levels remained at a relatively stable level indicating sustainable B cell neogenesis throughout life, which was in agreement with the findings reported previously (26, 39). The rapid decline of TREC and KREC levels in pediatrics emphasized the importance of establishing the age-specific reference intervals for PIDD diagnosis as the patients occurred mainly in this age range (16, 25).

TABLE 5 | Results of TREC and KREC levels in patients with primary immunodeficiency diseases.

Patient	Age	Diagnosis	Genetic diagnosis	TREC (copies/μL)	TREC (copies/10^6 cells)	KREC (copies/μL)	KREC (copies/10^6 cells)	Lymphocytes subset (cells/μL)	IgG/A/M (mg/dL)
P1	<1 y	SCID	RAG1 (c.322C>T & c.2095C>T)	*<LOD L*	*<LOD L*	*1 L*	*2,101 L*	CD3: 1,474, CD4: 531, CD8: 962; CD19: 226	IgG: 1,990, IgA: 33, IgM: 374
P2	<1 y	SCID; DLBCL	IL7RA (c.221+2T>A & c.361dupA)	*<LOD L*	*<LOD L*	*2 L*	*3,946 L*	CD3: 81, CD4: 7, CD8: 66; CD19: 700	IgG: 1,008, IgA:123, IgM: 181
P3	<1 y	X-SCID	IL2RG (c.694G>C)	*<LOD L*	*<LOD L*	*136*	*187,993*	CD3: 292, CD4: 31, CD8: 141, CD19: 910	IgG: 755, IgA: <7, IgM: 11
P4	<1 y	X-SCID	IL2RG (c.996C>T)	*<LOD L*	*<LOD L*	*142*	*61,424 L*	Not available	Not available
P5	<1 y	X-SCID	IL2RG (c.723T>G)	*<LOD L*	*<LOD L*	*43 L*	*26,158 L*	CD3: 175, CD4: 33, CD8: 136; CD19: 240	IgG: 326, IgA: <7, IgM: <5
P6	<1 y	DiGeorge Syndrome	22q11.21 deletion	*58 L*	*33,296 L*	138	*79,781 L*	CD3: 1,850, CD4: 1,200, CD8: 537; CD19: 892	IgG: 761, IgA: <10, IgM: <20
P7	5–8 y	APDS	PIK3CD (c.3061G>A)	*37 L*	*51,175 L*	*14 L*	*19,414 L*	CD3: 1,594, CD4: 831, CD8: 706; CD19: 98	IgG: 2,658, IgA: 77, IgM: 914
P8	1–4 y	XLA	BTK (c.1723G>C)	141	*61,379 L*	*<LOD L*	*<LOD L*	CD3: 2,212, CD4: 1,241, CD8: 674; CD19: 15	IgG: 336, IgA: <7, IgM: 18
P9	13–18 y	XLA	BTK (c.1535T>C)	87	76,075	*1 L*	*791 L*	CD3: 2,399, CD4: 927, CD8: 1,381; CD19: 8	IgG: <75, IgA: <10, IgM: 20
P10	<1 y	Agamma-globulinemia	Unknown	295	*88,500 L*	*<LOD L*	*<LOD L*	CD3: 7,845, CD4: 3,437, CD8: 5,116; CD19: 13	IgG: 726, IgA: <7, IgM: 27
P11	13–18 y	GATA2 deficiency; Monosomy 7	GATA2 (c.726_729del)	52	74,876	*<LOD L*	*<LOD L*	CD3: 1,169, CD4: 525, CD8: 599; CD19: 33	IgG: 766, IgA: 58, IgM: 106
P12	5–8 y	HIGM	CD40LG (c.761G>T)	175	83,734	109	51,992	CD3: 3,560, CD4: 1,995, CD8: 957; CD19: 767	IgG: 298, IgA: 61, IgM: 185

SCID, Severe Combined Immunodeficiency; DLBCL, Diffuse large B-cell lymphoma; X-SCID, X-linked SCID; XLA, X-linked Agammaglobulinemia; APDS, Activated PI3K-Delta Syndrome (APDS); HIGM, X-linked immunodeficiency with hyper-IgM syndrome; LOD, Limit of Detection; L, Below reference interval.

Our findings showed no significant differences in TREC levels between females and males, regardless of age, which echoed some studies that also found no differences between genders (32, 40, 41). However, several studies observed a higher TREC level in female adolescents and a slower decline of TREC levels in adult females compared to males (26, 28, 42), and Rechavi et al. reported a higher TREC level in female neonates than in male neonates (43). Interestingly, we observed a significantly higher level of KREC in females than males after 9–12 years (except for the 51–60 year age group), indicating a slower decline in KREC levels. This may be explained by the substantial immunomodulatory role of sex hormones on immune responses (44, 45). However, differences in the genetic backgrounds of ethnic groups may contribute to the contradictory findings. A larger sample size of infant healthy controls (<1 year) is needed to elucidate differences in TREC and KREC levels taking into account these factors. Moreover, TREC levels are maintained at very low levels or even undetectable in older age due to physiological decline of thymic function in the fifth and sixth decades of life, which might suggest that this assay is not suitable for adults over 50 years.

The diagnosis of PIDD is usually delayed until presentation of clinical symptoms, such as recurrent infections. The conventional tests for diagnosis of PIDD include total lymphocyte count, lymphocyte subset, immunoglobulin measurement, functional assays, and genetic analysis. The latter two remain the most important tests and are critical for diagnosis. TREC and KREC analysis is a fast, cost-effective and sensitive tool to for SCID screening and PIDD diagnosis, especially as only a small sample volume is needed for DNA extraction which minimizes harm to young age infants. It may not be feasible to perform all the conventional tests if sufficient blood is not available. The protocols of TREC and KREC assay varies in laboratories, and the genetic difference in populations may also contribute to the variation of cut-off in NBS programs in different countries. Therefore, the age- and ethnicity-matched TREC and KREC reference intervals reported in this study are crucial for the diagnosis of PIDD in young children and adolescents in the local population. Nevertheless, PIDD is a rare disease with around 140 cases diagnosed in Hong Kong in the past 30 years (46–48). Implementation of TREC (and KREC) based NBS program with local cut-off can greatly facilitate the early identification of severe PIDD patients. A quicker diagnosis allows earlier genotype-specific treatments (e.g., HSCT) prior to life-threatening infections, which offers a better outcome and improves the quality of life (49–51). This will also significantly reduce the costs of delayed diagnosis and treatment of related morbidities (52–54). We have shown a good correlation between the TREC results using our established assay with the CDC in-house method, indicating this assay is potentially applicable for the NBS program for severe PIDD.

Our TREC and KREC assay was able to correctly identify the 11 selected PIDD patients with aberrant T and/or B cell counts. The findings for SCID (RAG1 and IL2RG), XLA and GATA2 deficiency are in agreement with previous reports (55–59). Typical defect in IL7RA results in a phenotype of $T^{-}B^{+}NK^{+}$. However, the patient P2 in this study has concomitant EBV associated DLBCL. This might explain the low KREC level even the patient had a CD19 count of 600/μL and the circulating B cells might be dominated by the monoclonal expansion of cancer cells. Borte et al. also reported that patients with IL7RA defects could have variable KREC levels with the lowest level at ∼10 copies/μL (55). Recent pilot KREC NBS studies and our study have demonstrated the feasibility in detecting XLA and agammaglobulinemia (6, 60, 61). The identification of DGS has been challenging as the phenotypes vary from "partial" to "complete" reduction of thymus. Fronkova et al. reported that DGS patients had significantly lower TREC levels compared with controls, whereas TREC and KREC levels in most patients were within normal ranges (62). In our study, we detected a low TREC level and normal KREC level in a partial DGS patient compared with their age-matched 5^{th} percentile reference interval. Gul et al. and Liao et al. also found their DGS patients had TREC levels below the 5^{th} percentile and <90 copies, respectively (63, 64). Moreover, our assay was able to detect APDS caused by gain of function in PIK3CD which is a common variable immunodeficiency (CVID) with low TREC and KREC levels (65). As expected, HIGM patient with CD40LG defect has TREC and KREC levels within normal ranges (55). The application of 5^{th} percentile for TREC and KREC levels as the lower cut-off allows the detection of milder forms of T and B cells lymphopenia in PIDD diagnosis. Therefore, TREC and KREC assay is a valuable tool to facilitate the diagnosis of PIDD, in combination with other laboratory tests, such as lymphocyte subset and immunoglobulin measurement.

Since the first use of TREC level in determining thymic output by Douek et al., the units of measurement for TREC level have varied, including TREC/μg DNA of T cells, TREC/CD45RA+ T cell, TREC/10^5 CD4+ T cells, TREC/10^6 PBMCs, and TREC/mL or μL of blood (19, 28, 66). In this study, we measured the TREC, KREC, and β-actin (internal control) copy number using a multiplex RT-PCR assay and presented the TREC and KREC levels using two methods. We first aligned the formula used for the NBS program and expressed the levels in copies/μL blood (18, 19, 67). The TREC and KREC levels were then normalized with β-actin to calculate the levels in copies/10^6 cells (26). We showed a high correlation between the two units for both TREC and KREC levels. These calculation approaches avoided the need for flow cytometry to estimate the numbers of T cells or differential WBC counts, as the values may not be always available. The TREC and KREC levels in copies/μL of blood is suggested to be a better estimation of new lymphocyte maturation regardless of homeostatic cell replication (67). However, patients with lymphopenia due to immunosuppressive treatments may show "false positives" of low TREC and KREC copies per blood volume (6). In addition, patients with high T cells lymphoproliferation or monoclonal B cells proliferation may produce low TREC or KREC levels due to dilution in maturing cells (68). The TREC and KREC levels in copies/10^6 cells were normalized with the cell number in blood, which provides a more accurate evaluation of T and B cell function in such cases. However, elevated neutrophil numbers (e.g., during infection) may lead to under-estimation of TREC and KREC levels when using copies/10^6 cells. Therefore, TREC and KREC levels measured in both units should be

interpreted with care and other clinical information should be reviewed. Quantification of TREC levels can reflect the thymic output, as it is present in new and recent maturing lymphocytes, but low levels of TREC can still remain in some long-lived naïve cells, which can lead to over-estimation in the results (5, 69, 70).

Multiple methods have been applied for the quantitative measurement of TREC and/or KREC including RT-PCR, which is one of the most commonly used and cost-effective technologies (55). The introduction of KREC measurement offers an additional tool for detecting B cell lymphopenia. On the other hand, multiplex TREC, KREC and internal control in a single reaction using RT-PCR eliminates the variability associated with pipetting errors and allows accurate evaluation of TREC and KREC levels in a cost-effective manner (71). Pilot NBS study using TREC and KREC has also demonstrated improved diagnostic rates for severe PIDD (6, 60). Recent developments have also included the detection of exon 7 of SMN1 in a single reaction for the diagnostic testing of Spinal Muscular Atrophy (72–74). The use of digital PCR technology offers even better limits of detection and higher precision, which is particularly useful for samples with low copy numbers to help reduce the false positive rate (75–77). However, the drawback is the higher instrument costs (91, 93). Measurement of donor cell engraftment using chimerism analysis is a standard tool to determine immunity reconstitution (61). The TREC and KREC assay has been applied in post-HSCT monitoring of functional reconstitution of T and B cells (78, 79). The results obtained in this study could serve as age-matched reference intervals for the comparison of adult patients. Further study with more clinical samples for such applications is ongoing.

The findings in this study need to be interpreted with the following caveats. First, the number of archived samples for healthy infants (<1 year) was limited, which constitutes the major drawback of this study. This may be overcome by recruitment of more healthy subjects. This study also lacked adult PIDD samples for the evaluation of reference intervals in certain adult age groups. An international quality assurance program is available only for TREC measurements, thus an equivalent program for KREC measurements is warranted for standardizing assay performance across different laboratories (80).

CONCLUSION

To the best of our knowledge, we are first to report reference intervals of TREC and KREC levels from the largest sample size of healthy individuals in a Chinese population. Our study demonstrated that TREC and KREC levels decline with age, which is an important factor for the accurate measurement of TREC and KREC levels. This study generated age-matched reference values that allow us to interpret and compare results in samples of children, adolescents, and adults with suspected compromised immunity. The quantification of TREC and KREC levels simultaneously obtained by RT-PCR can be easily introduced into routine laboratory practice and is highly informative. PIDD including SCID, X-SCID, XLA, partial DGS, GATA2 deficiency, and APDS were successfully diagnosed using this assay. This assay is fast and cost-effective and the established age-specific reference intervals can be applied as a diagnostic tool for PIDD.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). Written, informed consent was obtained from the individuals and/or minors' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

The study was designed by JK, JH, SC, and PL. The TREC/KREC assays were developed by JH, SC, and IT. The samples were provided by PL, YL, DC, and VL. The TREC/KREC assays were performed by IT, PC, and EL. Data were collated by JH, SC, EL, and JK and analyzed by SC and JK. Statistical analyses were carried out by SC, EL, and JK. The manuscript was written by SC, PI, YL, and JK. The final manuscript was reviewed by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01411/full#supplementary-material

Supplementary Figure 1 | Dot Plot comparing the TREC copies/μL blood from measured results and expected results provided by the CDC for reference DBS specimens. A significant and strong positive correlation was observed between both methods ($r = 0.9176$, $p < 0.0001$).

REFERENCES

1. Jung D, Alt FW. Unraveling V(D)J recombination; insights into gene regulation. *Cell.* (2004) 116:299–311. doi: 10.1016/S0092-8674(04)00039-X
2. Livak F, Schatz DG. Identification of V(D)J recombination coding end intermediates in normal thymocytes. *J Mol Biol.* (1997) 267:1–9. doi: 10.1006/jmbi.1996.0834
3. Takeshita S, Toda M, Yamagishi H. Excision products of the T cell receptor gene support a progressive rearrangement model of the alpha/delta locus. *EMBO J.* (1989) 8:3261–70. doi: 10.1002/j.1460-2075.1989.tb08486.x
4. Ye P, Kirschner DE. Reevaluation of T cell receptor excision circles as a measure of human recent thymic emigrants. *J Immunol.* (2002) 168:4968–79. doi: 10.4049/jimmunol.168.10.4968
5. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med.* (2001) 79:631–40. doi: 10.1007/s001090100271
6. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterstrom 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) 37:51–60. doi: 10.1007/s10875-016-0347-5
7. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of kappa genes in human B cells. *Nature.* (1985) 316:260–2. doi: 10.1038/316260a0
8. van Zelm MC, van der Burg M, Langerak AW, van Dongen JJ. 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) 2:12. doi: 10.3389/fimmu.2011.00012
9. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med.* (2007) 204:645–55. doi: 10.1084/jem.20060964
10. Kong FK, Chen CL, Six A, Hockett RD, Cooper MD. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. *Proc Natl Acad Sci USA.* (1999) 96:1536–40. doi: 10.1073/pnas.96.4.1536
11. Livak F, Schatz DG. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. *Mol Cell Biol.* (1996) 16:609–18. doi: 10.1128/MCB.16.2.609
12. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, 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) 40:24–64. doi: 10.1007/s10875-020-00763-0
13. Rubin Z, Pappalardo A, Schwartz A, Antoon JW. Prevalence and outcomes of primary immunodeficiency in hospitalized children in the United States. *J Allergy Clin Immunol Pract.* (2018) 6:1705–10.e1. doi: 10.1016/j.jaip.2017.12.002
14. Modell V, Knaus M, Modell F, Roifman C, Orange J, Notarangelo LD, et al. Global overview of primary immunodeficiencies: a report from Jeffrey Modell Centers worldwide focused on diagnosis, treatment, and discovery. *Immunol Res.* (2014) 60:132–44. doi: 10.1007/s12026-014-8498-z
15. Lee PP, Lau YL. Improving care, education, and research: the Asian primary immunodeficiency network. *Ann NY Acad Sci.* (2011) 1238:33–41. doi: 10.1111/j.1749-6632.2011.06225.x
16. Lee PP, Lau YL. Primary immunodeficiencies: "new" disease in an old country. *Cell Mol Immunol.* (2009) 6:397–406. doi: 10.1038/cmi.2009.51
17. Reust CE. Evaluation of primary immunodeficiency disease in children. *Am Fam Physician.* (2013) 87:773–8.
18. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol.* (2005) 115:391–8. doi: 10.1016/j.jaci.2004.10.012
19. van der Spek J, Groenwold RH, van der Burg M, van Montfrans JM. TREC based newborn screening for severe combined immunodeficiency disease: a systematic review. *J Clin Immunol.* (2015) 35:416–30. doi: 10.1007/s10875-015-0152-6
20. van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal newborn screening for severe combined immunodeficiency (SCID). *Front Pediatr.* (2019) 7:373. doi: 10.3389/fped.2019.00373
21. Olbrich P, de Felipe B, Delgado-Pecellin C, Rodero R, Rojas P, Aguayo J, et al. A first pilot study on the neonatal screening of primary immunodeficiencies in Spain: TRECS and KRECS identify severe T- and B-cell lymphopenia. *Pediatr.* (2014) 81:310–7. doi: 10.1016/j.anpede.2014.08.003
22. 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. doi: 10.3389/fimmu.2018.00782
23. Palmer S, Albergante L, Blackburn CC, Newman TJ. Thymic involution and rising disease incidence with age. *Proc Natl Acad Sci USA.* (2018) 115:1883–8. doi: 10.1073/pnas.1714478115
24. Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol Immunol.* (2002) 38:841–8. doi: 10.1016/S0161-5890(01)00122-5
25. McCusker C, Upton J, Warrington R. Primary immunodeficiency. *Allergy Asthma Clin Immunol.* (2018) 14(Suppl 2):61. doi: 10.1186/s13223-018-0290-5
26. Sottini A, Serana F, Bertoli D, Chiarini M, Valotti M, Vaglio Tessitore M, et al. Simultaneous quantification of T-cell receptor excision circles (TRECs) and K-deleting recombination excision circles (KRECs) by real-time PCR. *J Vis Exp.* (2014) 52184. doi: 10.3791/52184
27. Atschekzei F, Ahmad F, Witte T, Jacobs R, Schmidt RE. Limitation of simultaneous analysis of t-cell receptor and kappa-deleting recombination excision circles based on multiplex real-time polymerase chain reaction in common variable immunodeficiency patients. *Int Arch Allergy Immunol.* (2016) 171:136–40. doi: 10.1159/000450950
28. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature.* (1998) 396:690–5. doi: 10.1038/25374
29. Chen X, Barfield R, Benaim E, Leung W, Knowles J, Lawrence D, et al. Prediction of T-cell reconstitution by assessment of T-cell receptor excision circle before allogeneic hematopoietic stem cell transplantation in pediatric patients. *Blood.* (2005) 105:886–93. doi: 10.1182/blood-2004-04-1405
30. Steffens CM, Al-Harthi L, Shott S, Yogev R, Landay A. Evaluation of thymopoiesis using T cell receptor excision circles (TRECs): differential correlation between adult and pediatric TRECs and naive phenotypes. *Clin Immunol.* (2000) 97:95–101. doi: 10.1006/clim.2000.4938
31. Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, et al. The influence of age on T cell generation and TCR diversity. *J Immunol.* (2005) 174:7446–52. doi: 10.4049/jimmunol.174.11.7446
32. Zhang L, Lewin SR, Markowitz M, Lin HH, Skulsky E, Karanicolas R, et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J Exp Med.* (1999) 190:725–32. doi: 10.1084/jem.190.5.725
33. Morinishi Y, Imai K, Nakagawa N, Sato H, Horiuchi K, Ohtsuka Y, et al. Identification of severe combined immunodeficiency by T-cell receptor excision circles quantification using neonatal guthrie cards. *J Pediatr.* (2009) 155:829–33. doi: 10.1016/j.jpeds.2009.05.026
34. Kamae C, Nakagawa N, Sato H, Honma K, Mitsuiki N, Ohara O, et al. Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin kappa-deleting recombination excision circles. *J Allergy Clin Immunol.* (2013) 131:1437–40 e5. doi: 10.1016/j.jaci.2012.10.059
35. Hasselbalch H, Jeppesen DL, Ersboll AK, Engelmann MD, Nielsen MB. Thymus size evaluated by sonography. a longitudinal study on infants during the first year of life. *Acta Radiol.* (1997) 38:222–7. doi: 10.1080/02841859709172053
36. Yekeler E, Tambag A, Tunaci A, Genchellac H, Dursun M, Gokcay G, et al. Analysis of the thymus in 151 healthy infants from 0 to 2 years of age. *J Ultrasound Med.* (2004) 23:1321–6. doi: 10.7863/jum.2004.23.10.1321
37. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD, et al. Thymic involution and immune reconstitution. *Trends Immunol.* (2009) 30:366–73. doi: 10.1016/j.it.2009.04.003
38. Verstegen RHJ, Aui PM, Watson E, De Jong S, Bartol SJW, Bosco JJ, et al. Quantification of T-Cell and B-cell replication history in aging, immunodeficiency, and newborn screening. *Front Immunol.* (2019) 10:2084. doi: 10.3389/fimmu.2019.02084

39. Shakerian L, Pourpak Z, Shamlou S, Domsgen E, Kazemnejad A, Dalili H, et al. Determining laboratory reference values of TREC and KREC in different age groups of iranian healthy individuals. *Iran J Allergy Asthma Immunol.* (2019) 18:143–52. doi: 10.18502/ijaai.v18i2.917
40. Arismendi MI, Kallas EG, Santos BA, Carneiro-Sampaio MM, Kayser C. Thymopoiesis and regulatory T cells in healthy children and adolescents. *Clinics.* (2012) 67:425–9. doi: 10.6061/clinics/2012(05)04
41. Geenen V, Poulin JF, Dion ML, Martens H, Castermans E, Hansenne I, et al. Quantification of T cell receptor rearrangement excision circles to estimate thymic function: an important new tool for endocrine-immune physiology. *J Endocrinol.* (2003) 176:305–11. doi: 10.1677/joe.0.1760305
42. Pido-Lopez J, Imami N, Aspinall R. Both age and gender affect thymic output: more recent thymic migrants in females than males as they age. *Clin Exp Immunol.* (2001) 125:409–13. doi: 10.1046/j.1365-2249.2001.01640.x
43. 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. doi: 10.3389/fimmu.2017.01448
44. Giefing-Kroll C, Berger P, Lepperdinger G, Grubeck-Loebenstein B. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell.* (2015) 14:309–21. doi: 10.1111/acel.12326
45. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol.* (2016) 16:626–38. doi: 10.1038/nri.2016.90
46. Leung D, Lee PPW, Lau YL. Review of a decade of international experiences in severe combined immunodeficiency newborn screening using t-cell receptor excision circle. *HK J Paediatr.* (2020) 25:30–41.
47. Luk ADW, Lee PP, Mao H, Chan KW, Chen XY, Chen TX, et al. Family History of early infant death correlates with earlier age at diagnosis but not shorter time to diagnosis for severe combined immunodeficiency. *Front Immunol.* (2017) 8:808. doi: 10.3389/fimmu.2017.00808.eCollection2017
48. Lee PP, Chan KW, Chen TX, Jiang LP, Wang XC, Zeng HS, et al. Molecular diagnosis of severe combined immunodeficiency–identification of IL2RG, JAK3, IL7R, DCLRE1C, RAG1, and RAG2 mutations in a cohort of Chinese and Southeast Asian children. *J Clin Immunol.* (2011) 31:281–96. doi: 10.1007/s10875-010-9489-z
49. Buckley RH, Schiff SE, Schiff RI, Markert L, Williams LW, Roberts JL, et al. Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med.* (1999) 340:508–16. doi: 10.1056/NEJM199902183400703
50. Castagnoli R, Delmonte OM, Calzoni E, Notarangelo LD. Hematopoietic stem cell transplantation in primary immunodeficiency diseases: current status and future perspectives. *Front Pediatr.* (2019) 7:295. doi: 10.3389/fped.2019.00295
51. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med.* (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
52. Gardulf A, Winiarski J, Thorin M, Heibert Arnlind M, von Dobeln U, Hammarstrom L, et al. Costs associated with treatment of severe combined immunodeficiency-rationale for newborn screening in Sweden. *J Allergy Clin Immunol.* (2017) 139:1713–6 e6. doi: 10.1016/j.jaci.2016.10.043
53. 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 Neonat Screen.* (2019) 5:28. doi: 10.3390/ijns5030028
54. Thomas C, Durand-Zaleski I, Frenkiel J, Mirallie S, Leger A, Cheillan D, et al. Clinical and economic aspects of newborn screening for severe combined immunodeficiency: DEPISTREC study results. *Clin Immunol.* (2019) 202:33–9. doi: 10.1016/j.clim.2019.03.012
55. Borte S, von Dobeln 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) 119:2552–5. doi: 10.1182/blood-2011-08-371021
56. 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) 27:70–7. doi: 10.1111/pai.12501
57. Serana F, Chiarini M, Zanotti C, Sottini A, Bertoli D, Bosio A, 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) 11:119. doi: 10.1186/1479-5876-11-119
58. Novakova M, Zaliova M, Sukova M, Wlodarski M, Janda A, Fronkova E, et al. Loss of B cells and their precursors is the most constant feature of GATA-2 deficiency in childhood myelodysplastic syndrome. *Haematologica.* (2016) 101:707–16. doi: 10.3324/haematol.2015.137711
59. Borte S, Wang N, Oskarsdottir S, von Dobeln U, Hammarstrom L. Newborn screening for primary immunodeficiencies: beyond SCID and XLA. *Ann NY Acad Sci.* (2011) 1246:118–30. doi: 10.1111/j.1749-6632.2011.06350.x
60. Wakamatsu M, Muramatsu H, Kataoka S, Okuno Y, Yoshimi S, Nakajima Y, et al. Utility of newborn screening for severe combined immunodeficiency and X-Linked agammaglobulinemia using TREC and KREC assays. *Blood.* (2019) 134:3604. doi: 10.1182/blood-2019-126669
61. Khan F, Agarwal A, Agrawal S. Significance of chimerism in hematopoietic stem cell transplantation: new variations on an old theme. *Bone Marrow Transplant.* (2004) 34:1–12. doi: 10.1038/sj.bmt.1704525
62. Fronkova E, Klocperk A, Svaton M, Novakova M, Kotrova M, Kayserova J, et al. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. *PLoS ONE.* (2014) 9:e114514. doi: 10.1371/journal.pone.0114514
63. Gul KA, Overland T, Osnes L, Baumbusch LO, Pettersen RD, Lima K, et al. Neonatal levels of T-cell Receptor Excision Circles (TREC) in patients with 22q11.2 deletion syndrome and later disease features. *J Clin Immunol.* (2015) 35:408–15. doi: 10.1007/s10875-015-0153-5
64. Liao HC, Liao CH, Kao SM, Chiang CC, Chen YJ. Detecting 22q11.2 deletion syndrome in newborns with low t cell receptor excision circles from severe combined immunodeficiency screening. *J Pediatr.* (2019) 204:219–24 e1. doi: 10.1016/j.jpeds.2018.08.072
65. Tsujita Y, Mitsui-Sekinaka K, Imai K, Yeh TW, Mitsuiki N, Asano T, et al. Phosphatase and tensin homolog (PTEN) mutation can cause activated phosphatidylinositol 3-kinase delta syndrome-like immunodeficiency. *J Allergy Clin Immunol.* (2016) 138:1672–80 e10. doi: 10.1016/j.jaci.2016.03.055
66. King JR, Hammarstrom L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol.* (2018) 38:56–66. doi: 10.1007/s10875-017-0455-x
67. Lorenzi AR, Patterson AM, Pratt A, Jefferson M, Chapman CE, Ponchel F, et al. Determination of thymic function directly from peripheral blood: a validated modification to an established method. *J Immunol Methods.* (2008) 339:185–94. doi: 10.1016/j.jim.2008.09.013
68. Ye P, Kirschner DE. Measuring emigration of human thymocytes by T-cell receptor excision circles. *Crit Rev Immunol.* (2002) 22:483–97. doi: 10.1615/CritRevImmunol.v22.i5-6.80
69. Hazenberg MD, Otto SA, Cohen Stuart JW, Verschuren MC, Borleffs JC, Boucher CA, et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med.* (2000) 6:1036–42. doi: 10.1038/79549
70. De Boer RJ, Perelson AS. Quantifying T lymphocyte turnover. *J Theor Biol.* (2013) 327:45–87. doi: 10.1016/j.jtbi.2012.12.025
71. Sottini A, Ghidini C, Zanotti C, Chiarini M, Caimi L, Lanfranchi A, et al. Simultaneous quantification of recent thymic T-cell and bone marrow B-cell emigrants in patients with primary immunodeficiency undergone to stem cell transplantation. *Clin Immunol.* (2010) 136:217–27. doi: 10.1016/j.clim.2010.04.005
72. Prior TW, Snyder PJ, Rink BD, Pearl DK, Pyatt RE, Mihal DC, et al. Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet A.* (2010) 152A:1608–16. doi: 10.1002/ajmg.a.33474
73. Taylor JL, Lee FK, Yazdanpanah GK, Staropoli JF, Liu M, Carulli JP, et al. Newborn blood spot screening test using multiplexed real-time PCR to simultaneously screen for spinal muscular atrophy and severe combined immunodeficiency. *Clin Chem.* (2015) 61:412–9. doi: 10.1373/clinchem.2014.231019
74. Czibere L, Burggraf S, Fleige T, Gluck B, Keitel LM, Landt O, et al. High-throughput genetic newborn screening for spinal muscular atrophy by rapid nucleic acid extraction from dried blood spots and 384-well qPCR. *Eur J Hum Genet.* (2020) 28:23–30. doi: 10.1038/s41431-019-0476-4
75. Tessitore MV, Sottini A, Roccaro AM, Ghidini C, Bernardi S, Martellosio G, et al. Detection of newly produced T and B lymphocytes by digital PCR in blood stored dry on nylon flocked swabs. *J Transl Med.* (2017) 15:70. doi: 10.1186/s12967-017-1169-9

76. Vidal-Folch N, Milosevic D, Majumdar R, Gavrilov D, Matern D, Raymond K, et al. A droplet digital pcr method for severe combined immunodeficiency newborn screening. *J Mol Diagn.* (2017) 19:755–65. doi: 10.1016/j.jmoldx.2017.05.011
77. Profaizer T, Slev P. A multiplex, droplet digital PCR assay for the detection of T-cell receptor excision circles and kappa-deleting recombination excision circles. *Clin Chem*. (2019) 66:229–38. doi: 10.1373/clinchem.2019.308171
78. Mensen A, Ochs C, Stroux A, Wittenbecher F, Szyska M, Imberti L, et al. Utilization of TREC and KREC quantification for the monitoring of early T- and B-cell neogenesis in adult patients after allogeneic hematopoietic stem cell transplantation. *J Transl Med.* (2013) 11:188. doi: 10.1186/1479-5876-11-188
79. Gaballa A, Sundin M, Stikvoort A, Abumaree M, Uzunel M, Sairafi D, et al. T Cell Receptor Excision Circle (TREC) monitoring after allogeneic stem cell transplantation; a predictive marker for complications and clinical outcome. *Int J Mol Sci.* (2016) 17:1705. doi: 10.3390/ijms17101705
80. Claustres M, Kozich V, Dequeker E, Fowler B, Hehir-Kwa JY, Miller K, et al. Recommendations for reporting results of diagnostic genetic testing (biochemical, cytogenetic and molecular genetic). *Eur J Hum Genet.* (2014) 22:160–70. doi: 10.1038/ejhg.2013.125

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Asymptomatic Infant With Atypical SCID and Novel Hypomorphic *RAG* Variant Identified by Newborn Screening: A Diagnostic and Treatment Dilemma

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Edited by:
Mikko Risto Juhana Seppänen, Helsinki University Central Hospital, Finland

Reviewed by:
Kohsuke Imai, Tokyo Medical and Dental University, Japan
David Buchbinder, Children's Hospital of Orange County, United States

***Correspondence:**
Jolan E. Walter
jolanwalter@usf.edu

†These authors share senior authorship

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Maria Chitty-Lopez[1], *Emma Westermann-Clark*[1], *Irina Dawson*[1], *Boglarka Ujhazi*[1], *Krisztian Csomos*[1], *Kerry Dobbs*[2], *Khuong Le*[2], *Yasuhiro Yamazaki*[2], *Amir A. Sadighi Akha*[3], *Deepak Chellapandian*[4], *Ben Oshrine*[4], *Luigi D. Notarangelo*[2], *Gauri Sunkersett*[4†], *Jennifer W. Leiding*[1†] *and Jolan E. Walter*[1,5*†]

[1] Division of Pediatric Allergy/Immunology, University of South Florida at Johns Hopkins All Children's Hospital, St. Petersburg, FL, United States, [2] Laboratory of Clinical Immunology and Microbiology, NIAID, National Institutes of Health, Bethesda, MD, United States, [3] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, United States, [4] Cancer and Blood Disorders Institute, Johns Hopkins All Children's Hospital, St. Petersburg, FL, United States, [5] Division of Pediatric Allergy and Immunology, Massachusetts General Hospital for Children, Boston, MA, United States

The T-cell receptor excision circle (TREC) assay detects T-cell lymphopenia (TCL) in newborns and is especially important to identify severe combined immunodeficiency (SCID). A spectrum of SCID variants and non-SCID conditions that present with TCL are being discovered with increasing frequency by newborn screening (NBS). Recombination-activating gene (RAG) deficiency is one the most common causes of classical and atypical SCID and other conditions with immune dysregulation. We present the case of an asymptomatic male with undetectable TRECs on NBS at 1 week of age. The asymptomatic newborn was found to have severe TCL, but normal B cell quantities and lymphocyte proliferation upon mitogen stimulation. Next generation sequencing revealed compound heterozygous hypomorphic *RAG* variants, one of which was novel. The moderately decreased recombinase activity of the *RAG* variants (16 and 40%) resulted in abnormal T and B-cell receptor repertoires, decreased fraction of CD3+ TCRVα7.2$^+$ T cells and an immune phenotype consistent with the *RAG* hypomorphic variants. The patient underwent successful treatment with hematopoietic stem cell transplantation (HSCT) at 5 months of age. This case illustrates how after identification of a novel *RAG* variant, *in vitro* studies are important to confirm the pathogenicity of the variant. This confirmation allows the clinician to expedite definitive treatment with HSCT in an asymptomatic phase, mitigating the risk of serious infectious and non-infectious complications.

Keywords: recombinase activating gene (RAG), newborn screening, SCID, asymptomatic infant, immunodeficiency, HSCT

INTRODUCTION

NBS for SCID was initially developed to identify affected infants, preferably at an asymptomatic stage, facilitating medical intervention before life-threatening complications arise (1–5). The assay is based on the absence or low count of T-cell receptor (TCR) excision circles (TREC), a by-product of TCR V (D) J rearrangement in the thymus during early T-cell development, and is universally available in the United States. Aside from SCID, low TREC detected on NBS also allows for the identification of other conditions associated with primary, secondary, and idiopathic forms of T-cell lymphopenia (TCL). Disorders associated with primary TCL include complete or partial DiGeorge syndrome, CHARGE syndrome, trisomy 21, and ataxia-telangiectasia. Examples of secondary TCL are post-surgical extravasation of fluid and lymphocytes, and gastrointestinal malformations resulting in lymphocyte loss. Finally, idiopathic TCL can occur in as many as 20% of screened cases with low TREC and these infants usually require close follow-up (6–8).

Without early intervention, such as HSCT, enzyme replacement therapy (ERT), or gene therapy (GT), a SCID patient is unlikely to survive beyond the first 2 years of life secondary to infections, failure to thrive, or immune dysregulation (4, 8). The great diversity of TCL-related conditions that can be identified via NBS for SCID in an otherwise asymptomatic child may create a degree of uncertainty regarding management strategies. This particularly applies to conditions where variants in SCID-related genes are discovered, but present with variable immune phenotypes.

Since the initiation of NBS for SCID, this condition is no longer defined by clinical symptoms in the majority of cases. Instead, variants of SCID are classified primarily by immune phenotype. The Primary Immunodeficiency Treatment Consortium (PIDTC) diagnostic algorithm subdivides SCID into three categories—typical SCID, atypical SCID, and Omenn syndrome (OS); patients are classified based on total T-cell enumeration, lymphocyte proliferation, presence of maternal T-cells, characteristic phenotypic features, and gene defects (9). SCID is further characterized by specific genotypes that can result in distinct immune phenotypes based on presence or absence of B and NK cells. X-linked interleukin 2 receptor gamma chain (IL2RG) deficiency, adenosine deaminase (ADA) deficiency, and RAG1/2 deficiencies are common genotypes in typical SCID that can be treated with HSCT. RAG1/2 deficiencies are also the most frequent genotypes to cause atypical SCID variants (4, 5).

There is an emerging category of combined immunodeficiencies (CID) with or without immune dysregulation resulting from hypomorphic variants of SCID genes. Partial RAG defects are an example of this phenomenon (10–13). In contrast with RAG1/2 null variants that typically manifest with a T-B- SCID phenotype, hypomorphic RAG deficiency has a broad spectrum of clinical presentations including OS, atypical SCID, combined immune deficiency with granuloma and/or autoimmunity (CID-G/AI), and TCL (13, 14). Characteristically, a patient with partial RAG deficiency and CID has a low naïve T-cell compartment, but relatively preserved B-cell quantities and immunoglobulin levels that may progress to B-cell lymphopenia and hypogammaglobulinemia with age (13). Patients with CID secondary to hypomorphic *RAG* variants often do not meet PIDTC criteria for SCID or atypical SCID. As the *RAG* genes are highly polymorphic and the clinical spectrum of RAG deficiencies is broad, specific functional assays and T cell receptor/B cell receptor (TCR/BCR) repertoire studies are required to establish the link between a novel *RAG* variant and the patient's immune phenotype.

We present an asymptomatic infant who had undetectable TREC on NBS for SCID and was found to carry compound heterozygous *RAG1* variants, resulting in an abnormal immune phenotype.

CASE PRESENTATION

The patient is a 1-week-old male newborn admitted with concern for SCID due to undetectable TREC on NBS (normal range >20 copies/microliter). The patient was conceived by *in vitro* fertilization and born at 35 weeks of gestation. He had no family history of immunodeficiency.

Initial Evaluation

Initial laboratory evaluation was notable for a $T^-/B^+/NK^+$ immune phenotype (**Table 1**). Due to concern for SCID, pharmacologic prophylaxis against infections was initiated per institutional protocol with acyclovir, fluconazole, and pentamidine, as well as intravenous immune globulins (IVIG). The mother was cytomegalovirus (CMV) seronegative and the patient had no detectable CMV viral load by PCR, hence breastfeeding was permitted. Further infectious evaluation confirmed undetectable serum viral loads of HIV, EBV, and adenovirus by PCR.

Subsequent evaluation at 1 and 3 months of age revealed that profound T cell lymphopenia (TCL) persisted (<300 cells/μl) and the proportion of naïve $CD45RA^+CD4^+$ T-cells was below normal (60%, normal >90%). Maternal engraftment was excluded by short tandem repeats (STR) analysis. A microarray resulted normal. The patient continued to maintain remarkable $CD4^+$ and $CD8^+$ lymphopenia; however, B and NK cell quantities remained within normal range (**Table 1**). After the initial IVIG dose, the patient was transitioned to subcutaneous immunoglobulin replacement therapy (IgRT), which maintained IgG levels within normal range. IgA level was consistently low (<7 mg/dL) and IgM level remained normal during this time (**Table 1**). Lymphocyte proliferation to the mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM) measured at 1 month of age by chemically labeled EdU (thymidine analog) incorporation method were normal. At 3 months of age, lymphocyte proliferation to PHA, concanavalin A (ConA), and PWM measured by radioactive thymidine incorporation method were normal as well (**Table 1**). TCR Vβ spectratyping was abnormal: of the 28 probes used for 23 TCR Vβ families and sub-families, one had no peak, 8/28 (29%) demonstrated oligoclonality (i.e., <5 peaks), and 15/28 (53%) showed a polyclonal, non-Gaussian distribution (**Figure 1B**).

Genomic DNA was subjected to target enrichment using hybridization capture with a custom bait pool and sequenced using Illumina sequencing chemistry. A validated bioinformatics

TABLE 1 | Laboratory assessments.

Age	Pre-HSCT			Post-HSCT			Age appropriate ranges	
	1 week old	1–2 months	3–4 months	12 months old	15 months old	17 months old	0–3 months old	12–18 months old
LYMPHOCYTE SUBSETS								
WBC	6,500 (L)	2,430		11,600	15,500 (H)	8,570	7,200–18,000	6,400–12,000
Lymphocyte	1,705 (L)	1,370 (L)	1,221 (L)	1,845 (L)	1,395 (L)	2,064	3,400–7,600	3,600–8,900
CD3+ absolute	261 (L)	202 (L)	203 (L)	858 (L)	529 (L)	1,182	2,500–5,500	2,100–6,200
CD4+ absolute	189 (L)	138 (L)	15 (L)	589 (L)	310 (L)	719	1,600–4,000	1,300–3,400
CD8+ absolute	60 (L)	55 (L)	45 (L)	137 (L)	125 (L)	312 (L)	560–1,700	620–2,000
CD19+ absolute	426	608	476	788	433	281 (L)	300–2,000	720–2,600
CD56+ absolute	967	523	538	207	417	574	170–1,100	180–920
CD4/CD45RA absolute and % of total CD4		82 (L) (60%)	87 (L) (60%)				1,200–3,700	1,000–2,900
CD8/CD45RA absolute and % of total CD8		40 (L) (72%)	37 (L) (82%)				450–1,500	490–1,700
IMMUNOGLOBULINS								
Unit: mg/dl								
IgG	1,000	608	570	514	605	569	251–906	345–1,213
IgM	27	27		49	57	120	20–87	43–173
IgA	<7 (<L)	<7 (L)		81	118	181 (H)	1.3–53	14–106
LYMPHOCYTE PROLIFERATION								
EdU (thymidine analog) incorporation								
Lymphocyte viability, LPM		(L) 64.5					>74.9	
PWM-induced, CD45		33					>4.4	
PWM-induced, CD3		42.1					>3.4	
PWM-induced, CD19		6.4					>3.8	
PHA-induced, CD45		75.1					>49.8	
PHA-induced, CD3		89.6					>58.4	
3H-thymidine incorporation								
PHA- induced, CMP			74,853		147,025	223,240	3,700–265,00	73,700–265,000
PHA- induced, SI			536		355	1,432		
Con-A-induced, CMP			61,045		82,251	92,041	6,100–283,00	46,100–283,000
Con-A-induced, SI			437		199	591		
PWM-induced, CMP			44,852		62,143	47,668	9,100–125,00	29,100–125,000
PWM-induced, SI			321		151	307		
NKC, Pct CYTOTOXICITY								
NKC, Pct cytotoxicity 50:1		25						
NKC, Pct cytotoxicity 25:1		24						
NKC, Pct cytotoxicity 12:1		15						
NKC, Pct cytotoxicity 6:1		11.5						
NKC, CD16/56 positive		27 (H)						

WBC, white blood cell count; PWM, pokeweed mitogen; PHA, phytohemagglutinin; Con-A, concanavalin A; NKC, natural killer cells; SI, stimulation index.

pipeline incorporating community standard and custom algorithms was used to simultaneously identify sequence changes and exonic deletions/duplications. Next generation sequencing identified two heterozygous missense variants in trans in *RAG1* (*RAG1* c.983G>A p.Cys328Tyr [C328Y] and *RAG1* c.1856C>T p.Pro619Leu [P619L]). The first variant had been previously published as a homozygous trait in a patient with OS and was also present in the patient's father, who was a heterozygous carrier (15). The second variant was a novel variant of uncertain significance (VUS); the patient's mother was a heterozygous carrier for this variant. These findings confirmed the compound heterozygous status of the patient.

Evaluation of Rag Variants by Population Database Frequency, *in silico* Analysis, and Recombinase Activity

RAG1 C328Y has not been linked to any specific ethnicity and has a low mean allele frequency (MAF) in heterozygous form 1/31,392 (3.2e-5) (gnomAD database, June 2020). This non-conservative amino acid substitution could potentially affect

A

RAG1 Variant	CliVar classification	Minor Allele frequency (MAF)* hetero/homozygous (dbSNP rs#)	Biochemical characteristics	Polyphen (P) / SIFT (S) predictions	Relative recombinase activity level
c.983 G>A p. Cys328Tyr [C328Y]	pathogenic	$3.18x10^{-5}$ / 0 rs121918571	missense amino acid substitution potentially affects secondary protein structure residues differ in polarity, charge, size	P: probably damaging S: deleterious	~16%*
c1856 C>T p.Pro619Leu [P619L]	Variant of uncertain significance (VUS)	$1.19x10^{-5}$ / 0 rs755059628	missense amino acid substitution likely to affect secondary protein structure residues differ in polarity, charge, size	P: probably damaging S: deleterious	~40%

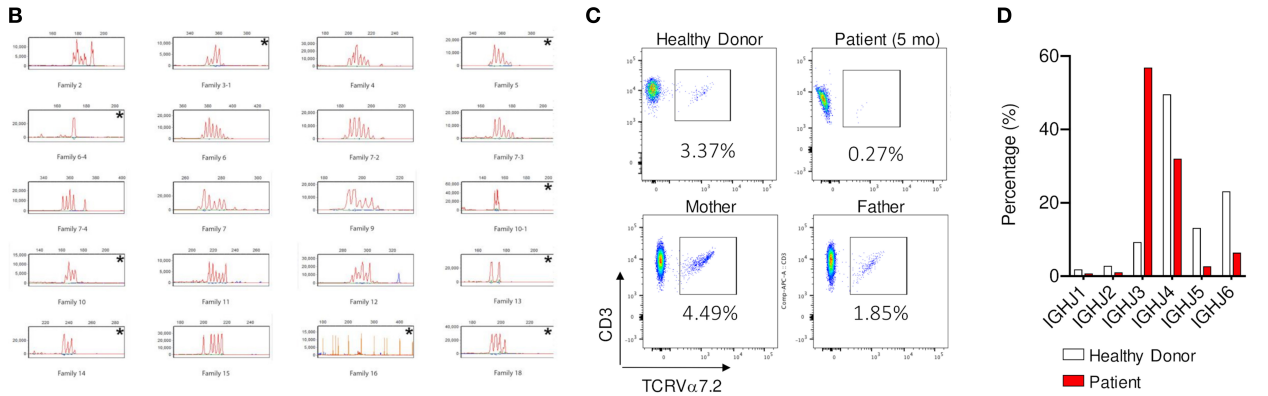

FIGURE 1 | (A) NGS identified two heterozygous missense variants in trans in *RAG1*. Relative recombination activity level of the two alleles [16% (ref 16) and 40% (novel variant, measured in this study)] predict an overall relative recombinase activity of 28% that is characteristic of CID-G/AI phenotype (*based on gnomAD 12/2019). **(B)** CD3+ T cells analyzed by spectratyping using 28 probes to measure CDR3 length variability in 23 TCRVβ families and sub-families. Families 2 to 18 are selected for representation. The following families demonstrated an oligoclonal distribution, i.e., <5 peaks: 3–1, 5, 6–4, 10–1, 10, 13, 14, and 18; family 16 had no peak. Each is identified with an * in the upper right corner. **(C)** Detection of Vα7.2 segment in CD3+ T lymphocytes by flow cytometry shows that the patient had near absence of CD3+T cells expressing the TCR Vα 7.2 chain [encoded by a distal V TCR locus] compared to healthy control and family members (measured in collaboration with the NIH). **(D)** Frequency of individual IGHJ gene usage of memory B cell receptor repertoire shows decrease in usage of the distal IGHJ5 and IGHJ6 gene elements in patient compared to age matched healthy donor.

secondary protein structure, as these residues differ in polarity, charge, size, and/or other properties. Polyphen and SIFT software programs predict this substitution as probably damaging and deleterious, respectively. *In vitro* relative recombinase activity determined by functional studies has previously shown that C328Y has reduced, but not absent, recombinase activity (16%) (16, 17). Therefore, this variant was established as a likely pathogenic hypomorphic variant.

The pathogenicity of the *RAG1* P619L variant has not previously been published or fully evaluated. Population studies detected this variant in South Asian populations with a MAF of 2/30,782 (6.5e-5) and a European cohort with a MAF of 1/113,661 (8.7e-6), with an overall MAF of 3/251,358 (1.19e-5) (gnomAD database, June 2020) (18). This non-conservative amino acid substitution is likely to affect secondary protein structure, as these residues also differ in polarity, charge, size, and/or other properties. Indeed, Polyphen and SIFT predict this variant as probably damaging and deleterious, respectively. Relative recombinase activity was previously unpublished for this variant and so was evaluated using a previously described platform (17). Relative recombinase activity of the *RAG1* P619L variant was reduced at 40%. In summary, based on the patient's clinical phenotype, repertoire studies, analysis of predicted genotype-phenotype implications, and relative recombinase activity measurement, it is likely that the *RAG1* P619L variant should be reclassified from a VUS to a likely pathogenic hypomorphic variant. Considering one variant had 16% predicted activity and the variant on the other allele 40% predicted activity, the calculated average recombinase activity is 28% (**Figure 1A**).

TCR and BCR Repertoire Data

TCR/BCR repertoire studies are an *in vivo* readout for deficiency in V (D) J recombination activity. In particular, reduced use of distal elements can reflect decreased capacity for *RAG* recombinase activity (19). In comparison to healthy controls, there was also a dramatic decline in usage of the distal *IGHJ5* and *IGHJ6* gene elements (BCR), suggesting an abnormal V (D) J recombination consistent with partial *RAG* deficiency (**Figure 1D**). Detection of Vα7.2 segment (TCR) in $CD3^+$ T lymphocytes and/or mucosa-associated invariant T-cells (MAIT) is a rapid method for evaluating decreased usage of distal TCRαV (*TRAV*) gene segments, which indicates a V (D) J recombination defect (20). Indeed, in our case we noted a decrease in $CD3^+$ $TCRV\alpha7.2^+$ lymphocytes compared to controls (0.27% vs. 1.85–4.49%) (**Figure 1C**). The TCR/BCR repertoire studies, recombinase activity assay, and specific flow cytometric testing for distal Vα7.2 element, all support a V (D) J recombination defect with partial RAG deficiency as the underlying cause of disease.

Additional Testing

Lastly, autoantibody testing targeting IFNα, IFNω, and IL-12 was performed by ELISA, as presence of these autoantibodies

have been associated with immune dysregulation in partial *RAG* deficiency (12, 21). These anti-cytokine antibodies were not detected in our patient. Increased natural killer (NK) cell cytotoxicity has been associated with increased rate of graft rejection after HSCT in RAG deficient patients (22). In our patient, NK cell cytotoxicity performed at 2 months of age was normal (**Table 1**).

Therapeutic Approach

The immune phenotype of our patient included absent TREC, TCL, normal T cell proliferation to mitogens, normal NK cell cytotoxicity, and oligoclonality with reduced relative recombinase activity. Based on his immune abnormalities, HSCT was considered as definitive treatment. The patient lacked an HLA-identical sibling, but several 10/10 HLA-matched unrelated donors (MUD) were available. The patient remained on anti-microbial prophylaxis and protective isolation, infection-free until transplant. At 5 months of age, he underwent HSCT with unmanipulated bone marrow from CMV negative 10/10 MUD (5.56 million CD34+ cells per kg of recipient body weight) with reduced intensity conditioning regimen of sub-myeloablative busulfan, fludarabine, and alemtuzumab, with a busulfan target AUC of 3,500–4,000 μM x min/L (23). Graft versus host disease (GVHD) prophylaxis included tacrolimus with a goal trough level of 10–15 ng/mL and a short-course of methotrexate given on days +1, +3, +6, and +11. The early post-HSCT period was without major complications except for *Enterobacter* bacteremia and *Clostridium difficile* colitis that resolved with antibiotics. The patient had platelet engraftment on day +22 and neutrophil engraftment on day +27 after HSCT. He demonstrated 100% donor chimerism in whole blood, myeloid, and NK lineages on day +27 after transplant and 100% donor T-cell chimerism on day +35.

Follow-Up

His post-transplant course was complicated by skin-only stage I GvHD that responded to systemic steroids and tacrolimus, and topical immunomodulating agents tacrolimus 0.03% ointment and hydrocortisone 1% cream. At 12 months of age, the patient had an absolute lymphocyte count of 1,895 cells/μL, with 858 T-cells/μL and 788 B-cell cells/μL. Over the past 2 years, he has maintained >90% donor T-cell chimerism, and 30–70% donor B-cell chimerism. The patient was weaned from IgRT 10 months after HSCT (**Table 1**). He has remained infection-free, off anti-microbial prophylaxis, has developed no signs of autoimmunity or immune dysregulation, and is thriving.

DISCUSSION

Our case exemplifies the diagnostic and treatment challenges for patients with severe TCL, SCID-related gene defects (i.e., partial *RAG* deficiency), and atypical immune phenotype. The patient's normal B-cell count and immunoglobulin levels except for IgA, higher preserved fraction of naïve CD4+ (60%) and CD8+ (72–82%) T-cells, and normal lymphocyte proliferation with mitogen stimulation and reduced relative recombinase activity of 28% are unusual for most variants of SCID.

While multiple classifications exist for SCID and related disorders that include guidance from PIDTC, European Society for Immunodeficiencies (ESID) and International Union of Immunological Societies (IUIS) (24), these criteria are not applicable to all patients with SCID. Our patient's absolute CD3 T cell count meets the threshold for typical SCID based on PIDTC criteria. However, lymphocyte proliferation is normal, whereas it is very low in typical SCID and leaky SCID/Omenn syndrome according to the PIDTC criteria. He does not meet ESID criteria for typical SCID either, since he was asymptomatic and did not have an affected family member (25). Because of the patient's severe TCL, he was sequestered from birth until HSCT to prevent infections.

Classical SCID with RAG deficiency typically causes a T-B- phenotype as null RAG activity will impede functional TCR/BCR development and thus jeopardizes T and B cell survival. In cases of hypomorphic variants with partially preserved recombinase activity, T and B cells may survive with a restricted TCR/BCR repertoire. *RAG* genes are also highly polymorphic; therefore, all novel variants require confirmation of pathogenicity. This can be done through BCR or TCR repertoire studies and/or *in vitro* recombination assays (17, 26, 27).

Among the repertoire studies available to establish the significance of a *RAG* variant, detection of TCRVα7.2 segment in CD3+ T lymphocytes and/or MAIT is the most rapid and clinically feasible method for evaluating reduced usage of distal *TRAV* segments, indicating a V (D) J recombination defect or impaired thymocyte survival. Notably, TCRVβ spectratyping reflects skewed repertoire based on CDR3 length, commonly seen in SCID variants, but does not reflect use of distal elements, therefore would not inform of V (D) J recombination defects.

Early diagnosis of patients with RAG deficiency is of high importance. If left undiagnosed and untreated, these patients develop severe and often fatal complications of immune dysregulation and/or infections (21, 28, 29). However, there are also reports of the same compound heterozygous *RAG* variants resulting in variable phenotype and disease severity. The cases of two siblings, one with CID and the other with mild T-cell lymphopenia (30), and two adults with the same genetic defect, but highly divergent phenotype (antibody deficiency with mild vs. multiple autoimmune conditions) illustrate this point [patients 12 and 13 from (14) cohort]. Anti-cytokine antibodies are associated with immune deficiency, autoimmunity and/or immune dysregulation, especially in patients with partial RAG deficiency with severe herpesvirus infections (12, 21). Although tested, our patient had not yet developed anti-cytokine antibodies, presumably due to his young age and lack of infectious triggers.

Patients with partial RAG deficiency including atypical SCID and CID phenotype are increasingly recognized in young children and adults after severe infections (13, 14, 21). As with SCID, multiple classification systems for primary CID disorders exist, complicating diagnostic and management decisions. Roifman et al. distinguished CID from SCID based on a total CD3+ T-cell count of >500/μl (31). However, our patient had considerably <500 T cells/μl. In the cohort study of 103 patients by Roifman et al., only 5 had a *RAG*-related

condition with OS or SCID phenotypes. All patients had low proliferation to mitogens and low TREC. Our patient also had low TREC but normal mitogens. The conclusions of the report implied that patients with partial RAG deficiency may be detected by NBS, further exemplified in our case. The 2019 ESID criteria for diagnosis of CID requires a symptomatic patient (infections, immune dysregulation) or history of affected family members with immune phenotype of 2 of the 4 parameters (low CD3, CD4 or CD8 T cells; low naïve CD4 and/or CD8 T cells; elevated γδT-cells; or reduced proliferation to mitogen or TCR stimulation) (25). Our patient meets the first two immunophenotypic criteria (note: γδT-cells were not measured) but not the clinical criteria as he was protected from infection by his environment and has no family history of this condition. The ESID 2019 criteria do not discuss underlying genetic defects for CID patients. The IUIS 2020 classification does list genetic defects for CID and does not include *RAG* deficiency (32) in the immune dysregulation group, where the CID-G/AI phenotype would best belong (24). The average recombinase activity of our patient (28%) is consistent with CID but not with typical SCID or OS (<10%) where there is typically lower activity (17, 26). Upon completing extensive immunological testing, our patient appeared to have an atypical SCID phenotype that shares several features with CID.

With the introduction of NBS for SCID, HSCT for asymptomatic SCID infants is increasingly utilized. Our patient had a unconventional immune phenotype with overlap between atypical SCID and CID that influenced the decision and design of the conditioning and transplant process. It is well-established that symptomatic patients with infectious and autoimmune complications, and CID immune phenotype secondary to *RAG* deficiency, do not respond well to immune modulation and ultimately require HSCT (13). However, there are no case studies nor established guidelines available on how to proceed with an asymptomatic infant with extreme TCL but normal T cell proliferation, and mostly normal humoral immunity. The decision to pursue immune reconstitution in hypomorphic *RAG* variants is controversial given the lack of HSCT studies in this particular population of TCL (33). Speckmann et al. published the therapeutic perspective of 51 pediatric patients (median age 9.6 years) with variants of symptomatic atypical SCID and CID characterized by infections and/or immune dysregulation and reduced, but not absent, T-cell immunity (34). The immune phenotype of these patients was not severe enough for an unambiguous early transplant decision. In this study, 29 (56%) of the 51 patients had mild disease, and six were transplanted for "prophylactic reasons" [i.e., family history or because the genetic diagnosis (DCLEREC1, LIGIV, IL2RG, NBN) was considered an HSCT indication].

Because severe T cell lymphopenia predisposes to serious infections, and hypomorphic *RAG* variants are linked to severe autoimmune and inflammatory complications in addition to infections, HSCT was performed electively at 5 months of age for our patient, taking into account our institutional experience and the paucity of data on timing for conditioning in this specific population. Conditioning regimens are increasingly used and streamlined for asymptomatic SCID, leaky SCID infants, and CID patients with *RAG* deficiency in the United States (4, 15, 33, 35, 36). Overall, using chemotherapy-based conditioning confers the advantage of facilitating T and B-cell reconstitution compared to unconditioned transplants in which B-cell dysfunction can persist with engraftment (37). The overactivated T and NK cell compartments should also be considered in the conditioning process and, in this case, inform the decision to use alemtuzumab and pre-transplant immunoablation (38).

Our case underscores the opportunity that SCID-NBS may have in capturing patients with overlapping immune phenotypes before the full spectrum of infectious and non-infectious complications develops. We also demonstrated that the pathogenicity of novel *RAG* variants requires high scrutiny with several methods. Awareness of the multiple presentations of immune deficiency with *RAG* variants is necessary for proper care of patients with this condition. HSCT can be considered for an asymptomatic infant with severe TCL but normal T cell proliferation and B cell number; however, further studies are needed to improve the knowledge of the natural course of the disease.

ETHICS STATEMENT

Written informed consent was obtained from the minor's legal guardian for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MC-L, EW-C, and ID conceived the presented idea. BU, KL, KD, KC, YY, and AASA performed functional assays and assisted with data interpretation. DC and BO verified the analytical methods used and reviewed the clinical information presented. LN, GS, JL, and JW encouraged to describe this patient's entity and clinical course in the context of internationally accepted guidelines and supervised the findings of this work. All authors discussed the results and contributed and agreed to the final manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Dvorak CC, Cowan MJ, Logan BR, Notarangelo LD, Griffith LM, Puck JM, 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) 33:1156–64. doi: 10.1007/s10875-013-9917-y
2. Pai S-Y, Logan BR, Griffith LM, Buckley RH, Parrott RE, Buckley RH, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *New Engl J Med*. (2014) 371:434–46. doi: 10.1056/NEJMoa1401177
3. Heimall J, Buckley RH, Puck J, Fleisher TA, Gennery AR, Haddad E, et al. Recommendations for screening management of late effects in patients with severe combined immunodeficiency after allogenic hematopoietic cell transplantation: a consensus statement from the second pediatric blood marrow transplant consortium international conference on late effects after pediatric HCT. *Biol Blood Marrow Transplant*. (2017) 23:1229–40. doi: 10.1016/j.bbmt.2017.04.026
4. Heimall J, Logan BR, Cowan MJ, Notarangelo LD, Griffith LM, Puck JM, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. *Blood*. (2017) 130:2718–27. doi: 10.1182/blood-2017-05-781849
5. Haddad E, Logan BR, Griffith LM, Buckley RH, Parrott RE, Prockop SE, et al. SCID genotype and 6-month posttransplant CD4 count predict survival and immune recovery. *Blood*. (2018) 132:1737–49. doi: 10.1182/blood-2018-03-840702
6. 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) 34:289–303. doi: 10.1007/s10875-014-0006-7
7. Kuo CY, Garcia-Lloret MI, Slev P, Bohnsack JF, Chen K. Profound T-cell lymphopenia associated with prenatal exposure to purine antagonists detected by TREC newborn screening. *J Allergy Clin Immunol Pract*. (2017) 5:198–200. doi: 10.1016/j.jaip.2016.09.028
8. Kobrynski LJ. Identification of nonand#x2013;severe combined immune deficiency T-cell lymphopenia at newborn screening for severe combined immune deficiency. *Ann Allergy Asthma Immunol*. (2019) 123:424–7. doi: 10.1016/j.anai.2019.08.006
9. 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. *J Allergy Clin Immunol*. (2014) 133:1092–8. doi: 10.1016/j.jaci.2013.09.044
10. Schuetz C, Huck K, Gudowius S, Megahed M, Feyen O, Hubner B, et al. An immunodeficiency disease with RAG mutations and granulomas. *New Engl J Med*. (2008) 358:2030–8. doi: 10.1056/NEJMoa073966
11. De Ravin SS, Cowen EW, Zarember KA, Whiting-Theobald NL, Kuhns DB, Sandler NG, et al. Hypomorphic Rag mutations can cause destructive midline granulomatous disease. *Blood*. (2010) 116:1263–71. doi: 10.1182/blood-2010-02-267583
12. Walter JE, Rosen LB, Csomos K, Rosenberg JM, Mathew D, Keszei M, et al. Broad-spectrum antibodies against self-antigens and cytokines in RAG deficiency. *J Clin Invest*. (2015) 125:4135–48. doi: 10.1172/JCI80477
13. Farmer JR, Foldvari Z, Ujhazi B, De Ravin SS, Chen K, Bleesing JJH, et al. Outcomes and treatment strategies for autoimmunity and hyperinflammation in patients with RAG deficiency. *J Allergy Clin Immunol Pract*. (2019) 7:1970–85.e4. doi: 10.1016/j.jaip.2019.02.038
14. Lawless D, Geier CB, Farmer JR, Lango Allen H, Thwaites D, Atschekzei F, et al. Prevalence and clinical challenges among adults with primary immunodeficiency and recombination-activating gene deficiency. *J Allergy Clin Immunol*. (2018) 141:2303–6. doi: 10.1016/j.jaci.2018.02.007
15. Villa A, Sobacchi C, Notarangelo LD, Bozzi F, Abinun M, Abrahamsen TG, et al. V (D) J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. *Blood*. (2001) 97:81–8. doi: 10.1182/blood.V97.1.81
16. Simkus C, Anand P, Bhattacharyya A, Jones MJ. Biochemical and folding defects in a RAG1 variant associated with Omenn syndrome. *J Immunol*. (2007) 179:8332–40. doi: 10.4049/jimmunol.179.12.8332
17. Lee YN, Frugoni F, Dobbs K, Walter JE, Giliani S, Gennery AR, et al. A systematic analysis of recombination activity and genotype-phenotype correlation in human recombination-activating gene 1 deficiency. *J Allergy Clin Immunol*. (2014) 133:1099–108. doi: 10.1016/j.jaci.2013.10.007
18. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature*. (2016) 536:285–91. doi: 10.1038/nature19057
19. IJspeert H, Driessen GJ, Moorhouse MJ, Hartwig NG, Wolska-Kusnierz B, Kalwak K, et al. Similar recombination-activating gene (RAG) mutations result in similar immunobiological effects but in different clinical phenotypes. *J Allergy Clin Immunol*. (2014) 133:1124–33. doi: 10.1016/j.jaci.2013.11.028
20. Berland A, Rosain J, Kaltenbach S, Allain V, Mahlaoui N, Melki I, et al. PROMIDISα: A T-cell receptor alpha signature associated with immunodeficiencies caused by V (D) J recombination defects. *J Allergy Clin Immunol*. (2019) 143:325–34.e2. doi: 10.1016/j.jaci.2018.05.028
21. Goda V, Malik A, Kalmar T, Maroti Z, Patel B, Ujhazi B, et al. Partial RAG deficiency in a patient with varicella infection, autoimmune cytopenia, anticytokine antibodies. *J Allergy Clin Immunol Pract*. (2018) 6:1769–71.e2. doi: 10.1016/j.jaip.2018.01.015
22. Gennery A. Recent advances in understanding RAG deficiencies. *F1000Res*. (2019) 8. doi: 10.12688/f1000research.17056.1
23. Gungor T, Teira P, Slatter M, Stussi G, Stepensky P, Moshous D, et al. Reduced-intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: a prospective multicentre study. *Lancet*. (2014) 383:436–48. doi: 10.1016/S0140-6736(13)62069-3
24. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. *J Clin Immunol*. (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8
25. Seidel MG, Kindle G, Gathmann B, Quinti I, Buckland M, van Montfrans J, et al. The European Society for Immunodeficiencies (ESID) registry working definitions for the clinical diagnosis of inborn errors of immunity. *J Allergy Clin Immunol Pract*. (2019) 7:1763–70. doi: 10.1016/j.jaip.2019.02.004
26. Lee YN, Frugoni F, Dobbs K, Tirosh I, Du L, Ververs FA, et al. Characterization of T and B cell repertoire diversity in patients with RAG deficiency. *Sci Immunol*. (2016) 1:eaah6109. doi: 10.1126/sciimmunol.aah6109
27. Bulkhi AA, Dasso JF, Schuetz C, Walter EJ. Approaches to patients with variants in RAG genes: from diagnosis to timely treatment. *Expert Rev Clin Immunol*. (2019) 15:1033–46. doi: 10.1080/1744666X.2020.1670060
28. Dutmer CM, Asturias EJ, Smith C, Dishop MK, Schmid DS, Bellini WJ, et al. Late onset hypomorphic RAG2 deficiency presentation with fatal vaccine-strain VZV infection. *J Clin Immunol*. (2015) 35:754–60. doi: 10.1007/s10875-015-0207-8
29. Geier CB, Farmer JR, Foldvari Z, Ujhazi B, Eibl MM, Wolf HM, et al. Vasculitis as a major morbidity factor in patients with hypomorphic RAG mutations. *J Allergy Clin Immunol*. (2019) 143:AB116. doi: 10.1016/j.jaci.2018.12.353
30. Schuetz C, Pannicke U, Jacobsen EM, Burggraf S, Albert MH, Honig M, et al. Lesson from hypomorphic recombination-activating gene (RAG) mutations: why asymptomatic siblings should also be tested. *J Allergy Clin Immunol*. (2014) 133:1211–5. doi: 10.1016/j.jaci.2013.10.021
31. Roifman CM, Somech R, Kavadas F, Pires L, Nahum A, Dalal I, et al. Defining combined immunodeficiency. *J Allergy Clin Immunol*. (2012) 130:177–83. doi: 10.1016/j.jaci.2012.04.029
32. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, 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) 40:65. doi: 10.1007/s10875-020-00763-0
33. John T, Walter JE, Schuetz C, Chen K, Abraham RS, Bonfim C, et al. Unrelated hematopoietic cell transplantation in a patient with combined immunodeficiency with granulomatous disease and autoimmunity secondary to RAG deficiency. *J Clin Immunol*. (2016) 36:725–32. doi: 10.1007/s10875-016-0326-x
34. Speckmann C, Doerken S, Aiuti A, Albert MH, Al-Herz W, Allende LM, et al. A prospective study on the natural history of patients with profound combined immunodeficiency: an interim analysis. *J Allergy Clin Immunol*. (2017) 1391302–10.e4. doi: 10.1016/j.jaci.2016.07.040
35. Delmonte OM, Schuetz C, Notarangelo DL. RAG deficiency: two genes, many diseases. *J Clin Immunol*. (2018) 38:646–55. doi: 10.1007/s10875-018-0537-4

36. Westermann-Clark E, Grossi A, Fioredda F, Giardino S, Cappelli E, Terranova P, et al. RAG deficiency with ALPS features successfully treated with TCRαβ/CD19 cell depleted haploidentical stem cell transplant. *Clin Immunol.* (2018) 187:102–3. doi: 10.1016/j.clim.2017.10.012
37. Castagnoli R, Delmonte OM, Calzoni E, Notarangelo DL. Hematopoietic stem cell transplantation in primary immunodeficiency diseases: current status and future perspectives. *Front Pediatr*. (2019) 7:295. doi: 10.3389/fped.2019.00295
38. Dobbs K, Tabellini G, Calzoni E, Patrizi O, Martinez P, Giliani SC, et al. Natural killer cells from patients with recombinase-activating gene and non-homologous end joining gene defects comprise a higher frequency of CD56 (bright) NKG2A(+ + +) cells, and yet display increased degranulation and higher perforin content. *Front Immunol.* (2017) 8:798. doi: 10.3389/fimmu.2017.00798

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The Landscape of Severe Combined Immunodeficiency Newborn Screening in the United States in 2020: A Review of Screening Methodologies and Targets, Communication Pathways, and Long-Term Follow-Up Practices

Ruthanne Sheller[1], Jelili Ojodu[1], Emma Griffin[1], Sari Edelman[1], Careema Yusuf[1], Trey Pigg[1], Alissa Huston[2], Brian Fitzek[2], John G. Boyle[2] and Sikha Singh[1]*

[1] Newborn Screening and Genetics Department, Association of Public Health Laboratories, Silver Spring, MD, United States, [2] Immune Deficiency Foundation, Towson, MD, United States

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Jolan Eszter Walter,
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Sheba Medical Center, Israel

***Correspondence:**
Ruthanne Sheller
ruthanne.sheller@aphl.org

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Severe combined immunodeficiency (SCID) is T cell development disorders in the immune system and can be detected at birth. As of December 2018, all 53 newborn screening (NBS) programs within the United States and associated territories offer universal screening for SCID. The Association of Public Health Laboratories (APHL), along with the Immune Deficiency Foundation (IDF), surveyed public health NBS system laboratory and follow-up coordinators regarding their NBS program's screening methodologies and targets, protocols for stakeholder notifications, and long-term follow-up practices. This report explores the variation that exists across NBS practices, revealing needs for efficiencies and educational resources across the NBS system to ensure the best outcomes for newborns.

Keywords: severe combined immunodeficiency, newborn screening (NBS), United States, communication pathways, long-term follow-up, education

INTRODUCTION

In the United States, newborn screening (NBS) is a state public health service designed to identify individuals in a population at high risk of certain congenital or heritable disorders. With approximately four million newborns screened for at least 30 primary conditions every year, NBS is recognized as one of the most successful public health programs in the US (1).

While the Health and Human Services (HHS) Federal Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC) recommends disorders to be included on the Recommended Uniform Screening Panel (RUSP), each state determines the specific disorders for which it screens. In the United States, there are significant variations in geographic, population, and demographic characteristics within and between states. As such, each state NBS system applies

independent criteria to determine the timeline with which they will implement NBS for newly added RUSP conditions (2).

In May of 2010, severe combined immunodeficiency (SCID) was added to the RUSP. SCID are T-cell developmental disorders in the immune system, affecting approximately one in 58,000 births in the United States (3). Screening also identifies one in 20,000 newborns who have non-SCID T-cell lymphopenia (TCL) (4). Newborns with SCID (and TCL) appear healthy in the neonatal period, but are extremely vulnerable to infection. Exposure to common infections and live vaccines is life threatening unless timely treatment is provided in infancy. NBS contributes to optimized health outcomes for newborns with SCID through earlier detection, diagnosis, and treatment (5).

The addition of SCID to NBS panels posed both opportunities and challenges to NBS programs, requiring programs to integrate new screening technology within their laboratories, to train NBS personnel on this technology, and to establish clinical referral networks (6). As of December 2018, all 53 NBS programs within the United States and associated territories offer SCID NBS through the utilization of dried blood spot cards (**Supplementary Document**) (4, 7).

While the primary targets of SCID NBS are typical SCID, leaky SCID, and Omenn syndrome, additional conditions with low or absent T-cell numbers have been detected as secondary targets (5). NBS programs continue to partner with clinicians and the Newborn Screening Technical assistance and Evaluation Program (NewSTEPs) to collect SCID case-level data categorized by public health surveillance case definitions. These surveillance case definitions allow for consistent public health diagnoses and provide an estimate of the true birth prevalence of disorders identified by NBS (8).

In order to explore the variation that exists across SCID NBS practices in the US, the Association of Public Health Laboratories (APHL), in partnership with the Immune Deficiency Foundation (IDF), developed and fielded a survey to 53 NBS programs. The results revealed areas for addressing communication and follow-up inefficiencies as well as educational needs.

MATERIALS AND METHODS

In 2018, the IDF began a collaboration with APHL to implement the SCID Compass Program (9). Specifically, APHL provides technical assistance, trainings and resources to support SCID NBS. A component of the SCID Compass Program is to gain a clearer understanding of the disparate landscape of SCID NBS across all states and territories in the US. In doing so, APHL and IDF developed a web-based survey instrument using the Qualtrics software platform consisting of yes/no, open-ended, and multiple choice questions (**Supplementary Document**). This survey assessed NBS programs' laboratory methodologies and targets, protocols for stakeholder notifications, and long-term follow-up practices as they pertain to SCID.

The survey was distributed to laboratory and follow-up coordinators in all 50 states, as well as the District of Columbia, Guam, and Puerto Rico (N = 53). Survey respondents were encouraged to reach out to their respective laboratory or follow-up counterparts to submit collaborative responses, and were requested to complete one survey per state.

Over the course of 6 weeks, APHL staff followed-up with NBS programs *via* email and phone with reminders. Surveys were submitted electronically through the Qualtrics software platform. The following software programs supported subsequent survey data analysis efforts: Microsoft Excel, Microsoft Power BI, and Python.

Supplementary data was also received from the NewSTEPs data repository. NewSTEPs is a national NBS resource center designed to provide data, technical assistance and training to NBS programs and assist states with quality improvement initiatives. NewSTEPs provides a centralized and secure online national data repository designed to collect comprehensive data on US NBS programs, inclusive of state profile data, confirmed cases and quality indicators. State profile data, including the screening methodologies and targets used in this report, is public facing. Most data elements are summarized and reported in real time on the NewSTEPs website and data repository in the form of interactive Tableau maps, tables and reports (1).

The survey had four sections: 1) Screening Methodologies and Targets, 2) Communication Pathways, 3) Education, and 4) Long-Term Follow-Up. The results from each section are described below.

RESULTS

APHL received 50 completed surveys (n = 50/53; 94% response rate) from laboratory and follow-up coordinators, representing 49 states and 1 US territory over the course of 6 weeks.

Screening Methodologies and Targets

For the screening methodologies and targets section of the survey (**Supplementary Document**), respondents were requested to review their NBS program's information that had been submitted to the NewSTEPs Data Repository and indicate if any edits or updates were needed. Respondents who selected "yes" to edits needed were asked to identify their screening methods and targets for first- as well as for second-tier screening.

For first-tier screening for SCID, all programs use T-cell receptor excision circle (TREC) as a screening marker; 90% of NBS programs (n = 45/50) opted to implement SCID NBS using a laboratory-developed real-time polymerase chain reaction (PCR) method. Ten percent of the NBS programs (n = 5/50) elected to use a U.S. Food and Drug Administration (FDA)-approved end-point PCR method, the PerkinElmer EnLite Neonatal TREC Kit, which received approval in December 2014 (10).

Twenty percent of NBS programs (n = 10/50) reported performing second-tier screening for SCID. All ten of these programs reported using TREC as their marker for second-tier screening. One of these NBS programs contracts with an external laboratory to perform second-tier screening. One other NBS

program noted that their second-tier screening involved retesting specimens using their first-tier method in triplicate.

NBS programs were then asked if they multiplex SCID NBS with Spinal Muscular Atrophy (SMA) screening, both of which could employ the same molecular technique. At the time, this survey was issued, 18 NBS programs were offering universal SMA NBS. Of the 18 NBS programs offering SMA screening, 67% of these NBS programs (n = 12/18) reported multiplexing with SMA.

Communication Pathways

Respondents were asked to identify who (primary care physician (PCP)/other provider, immunology consultant, public health nurse, family, hospital or other submitter, other) they inform about an out-of-range newborn screen for SCID, as well as the method used to contact those stakeholders (telephone, letter, certified letter, fax, email, text, other). Respondents were able to select all of the responses that applied for questions under this section.

Fifty NBS programs (N = 50) reported on the stakeholders they notify about out-of-range screens for SCID. Eighty-eight percent (n = 44/50) of these NBS programs reported notifying PCP s/other providers of out-of-range screens. Seventy-eight percent (n = 39/50) notified immunology consultants, 30% (n = 15/50) notified hospitals or other submitters, 12% (n = 6/50) notified family, and 8% (n = 4/50) notified public health nurses. Twenty-eight percent of NBS programs (n = 14/50) indicated "other," listing genetic coordinators, genetic referral centers, department of health coordinators, and midwives as other stakeholders that may be notified of an out-of-range SCID NBS screen. Twelve percent of NBS programs (n = 6/50) selected family, clarifying that families were only contacted if they were unable to get in touch with a PCP/other provider (**Figure 1**).

Eighty-four percent of NBS programs (n = 42/50) reported that they notified at least two groups of stakeholders of out-of-range SCID newborn screens, with 42% (n = 21/50) notifying two contacts, 26% (n = 13/50) notifying three contacts, 14% (n = 7/50) notifying four contacts, and 2% (n = 1/50) notifying five contacts. Sixteen percent of NBS programs (n = 8/50) only notified one stakeholder about an out-of-range SCID newborn screen (**Figure 2**).

Figure 3 displays the various combinations of stakeholders that were notified of out-of-range screens. Primary care providers and immunologists were the most commonly notified stakeholders (n =16). Primary care providers, immunology consultants and hospitals/submitters were the second group of stakeholders most commonly notified (n = 6).

One-hundred percent of NBS programs (N = 50) reported the methods they utilize to notify stakeholders of an out-of-range SCID NBS screen. Ninety-eight percent of NBS programs (n = 49/50) utilized phones, 76% (n = 38/50) sent faxes, 40% (n = 20/50) email, 32% letter (n = 16/50), and 4% certified letter (n = 2/50). Three NBS programs (n = 3/50) noted the utilization of secure electronic systems to share results under "other".

Forty percent of NBS programs (n = 20/50) reported they utilized three different contact methods to notify stakeholders of out-of-range SCID NBS screens. Thirty percent of NBS programs (n = 15/50) utilized two contact methods, 14% (n = 7/50) NBS programs utilized either one contact method or four contact methods, and 2% (1/50) utilized five contact methods.

Figure 4 displays the various combinations of contact methods that were utilized to notify stakeholders of out-of-range SCID NBS screens. Phone and fax (n = 10) were the

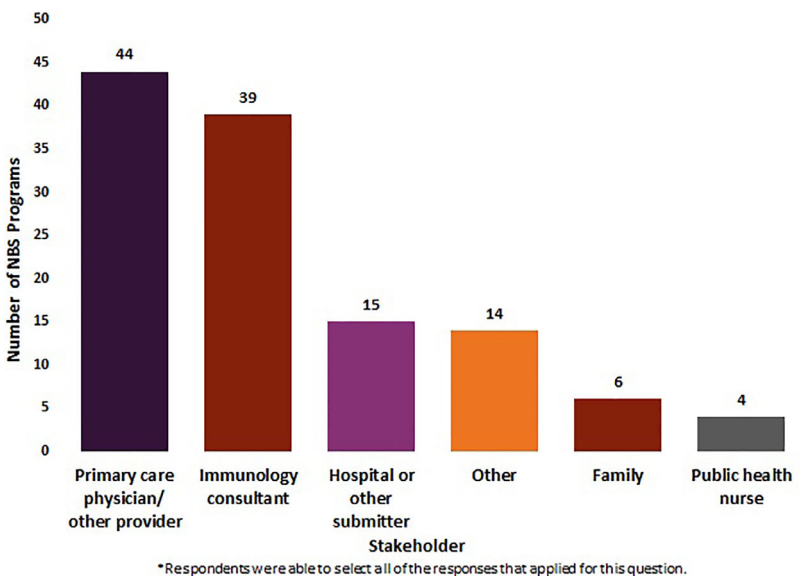

FIGURE 1 | Stakeholders notified by newborn screening programs about an out-of-range screen for SCID (January 2020) (N = 50).

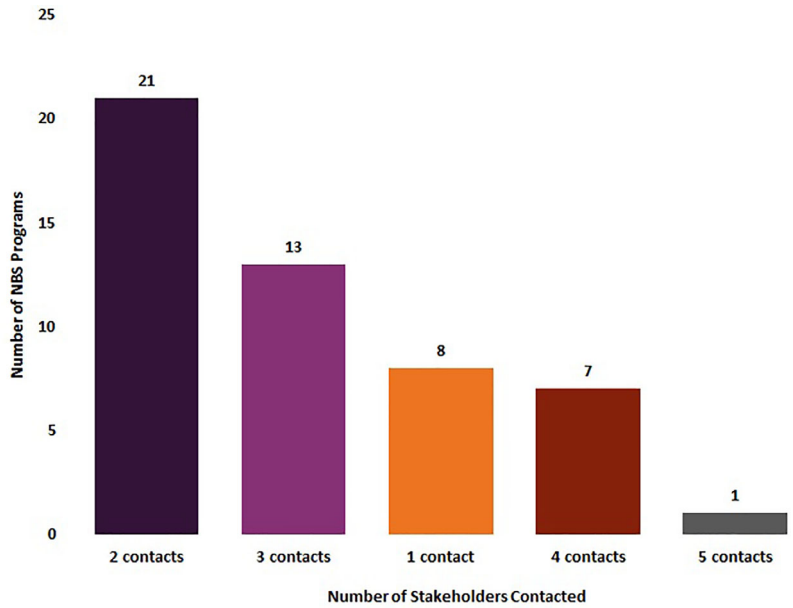

FIGURE 2 | Number of stakeholders notified by newborn screening programs about an out-of-range screen for SCID (January 2020) (N = 50).

most commonly used notification methods, followed by phone, fax, and email (n = 9) and phone, letter, and fax (n = 9).

Education

Respondents were asked if they had a targeted education plan for health care providers, parents, and/or for the general public. Sixty-six percent of NBS programs (n = 33/50) reported they had a targeted education plan for health care providers. Sixty-three percent of NBS programs (n = 31/49) indicated they had a targeted education plan for parents and 23% (n = 10/43) reported that they had a targeted education plan for the general public. Twenty-one percent of NBS programs (n = 9/43) reported they maintained educational plans for all three stakeholder groups.

Long-Term Follow-Up

Fifty-four percent of NBS programs (n = 27/50) reported that they do not follow patients after they have received a confirmed SCID diagnosis, with 4% of these NBS programs (n = 2/50) noting that they plan to initiate this practice in the coming year. Sixteen percent of NBS programs (n = 8/50) reported that they follow patients after they have received a confirmed diagnosis, from an age range of one year through 21 years (median = 10 years). Thirty-percent of NBS programs (n = 15/50) selected "other" as a response, with many NBS programs noting that once a patient receives a diagnosis, the patient is then followed by an immunologist, genetic specialist, clinical care center, social workers, or public health program such as the Children and Youth with Special Healthcare Needs Program within their state.

Eighty-two percent of NBS programs (N = 41/50) reported the elements for which they collected data on from patients who received an out-of-range SCID screen. Respondents were able to select all of the responses that applied for question. Seventy-eight percent of NBS programs (n = 32/41) collected where the patient is currently being seen by a specialty provider, 63% (n = 26/41) collected whether the patient is currently being seen by a PCP, 44% (n = 18/41) collected contact information of current PCP, 34% (n = 14/41) collected updated patient clinical data, 34% (n = 14/41) collected current patient treatment regimen, 27% (n = 11/41) collected date of last visit with the specialty provider, 17% (n = 7/41) collected patient's developmental progress, 12% (n = 5/41) collected changes to treatment regimen, and 5% (n = 2/41) collected data of the last visit with the PCP. Fifty-one percent of NBS programs selected "other" (n = 21/41), noting additional data elements that were collected such as treatments and interventions (n = 2), informal updates (n = 3), specialist referral follow-up (n = 5), and clinical data and test results (n = 9) (**Figure 5**).

Sixty-six percent of NBS programs (N = 33/50) reported how collected data on patients who received abnormal SCID screens was utilized. Respondents were able to select all of the responses that applied for question. Seventy percent of NBS programs (n = 23/33) reported data was used to track the number of patients lost to follow-up. Fifty-eight percent of NBS programs (n = 19/33) indicated that data was used to track the clinical outcomes of patients. Thirty-three percent of NBS programs (n = 11/33) used data to assess the needs of patients/families for services. Twenty-seven percent of NBS Programs (n = 9/33) utilized data collected to evaluate the performance of specialty providers (physicians, nurses, and allied health professionals). Six percent of NBS programs (n = 2/33) used collected data to conduct research, such as cost-benefit analysis of screening. NBS programs also reported that data was used to ensure timely follow-up and

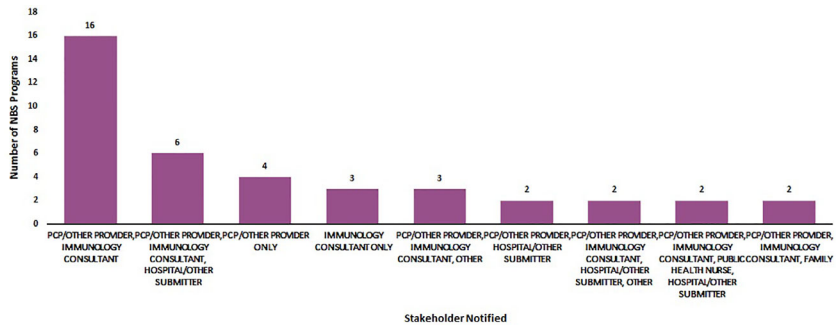

FIGURE 3 | Most commonly notified stakeholders about an out-of-range newborn screen for SCID (January 2020) (N = 50).

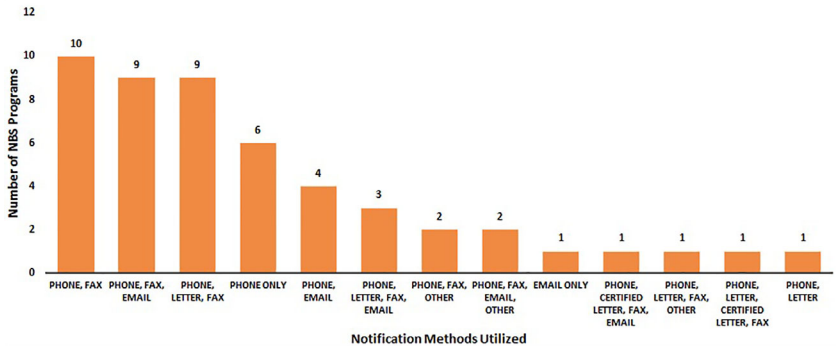

FIGURE 4 | Most common notification methods regarding an out-of-range newborn screen for SCID (January 2020) (N = 50).

treatment initiation, track the number of out-of-range, inconclusive, false positive, and false negative screens and associated diagnoses, and for broader publication reporting (i.e., Healthy People 2020) (11).

Seventy percent of NBS programs (n = 33/47) stated that the data elements listed above were entered into an electronic system or database. Thirty percent of NBS programs (n = 14/47) selected "no" in response to this question.

Respondents were asked to write in the name(s) of which electronic database/system they used. The electronic databases/systems reported can be stratified into six categories (N = 33): custom (n = 13), Neometrics (n = 8), PerkinElmer (n = 7), NewSTEPs (n = 5), Maven (n = 2), and StarLIMS (n = 1) (**Figure 6**). Fifteen percent of these NBS programs (n = 5/33) noted the utilization of multiple electronic databases or systems for data collection.

DISCUSSION

NBS identifies the risk for SCID in newborns by quantifying TRECs, which are produced during the normal development of T-cells. Low TREC levels facilitate the identification of patients with SCID and other serious medical conditions associated with low T-cell numbers (12). To detect TREC levels, state NBS programs have the option of utilizing a vendor kit based on end-point PCR, or a laboratory developed test (LDT) performing real-time PCR (6).

There are various benefits and limitations to both methods. The vendor kit provides ready quality assurance and quality control support; however, it was not made available to NBS programs until it was approved by the U.S. Food and Drug Administration (FDA) in December of 2014 (10).

Alternatively, LDTs can be more cost-effective and faster to implement. Many programs chose to adapt an automated *in situ* dried blood spot real time PCR TREC assay developed by the Centers for Disease Control and Prevention (CDC). This approach is limited to specific instrumentation (real-time PCR instruments with 96-well format) (6).

LDTs also offer screening flexibility when considering the addition of new conditions such as SMA. With 90% of NBS programs (n = 45/50) reporting they used LDTs, this flexibility may prove to be useful as they consider adding SMA to their state NBS panels. However, some NBS programs may also choose not to multiplex due to their individual program's needs (6).

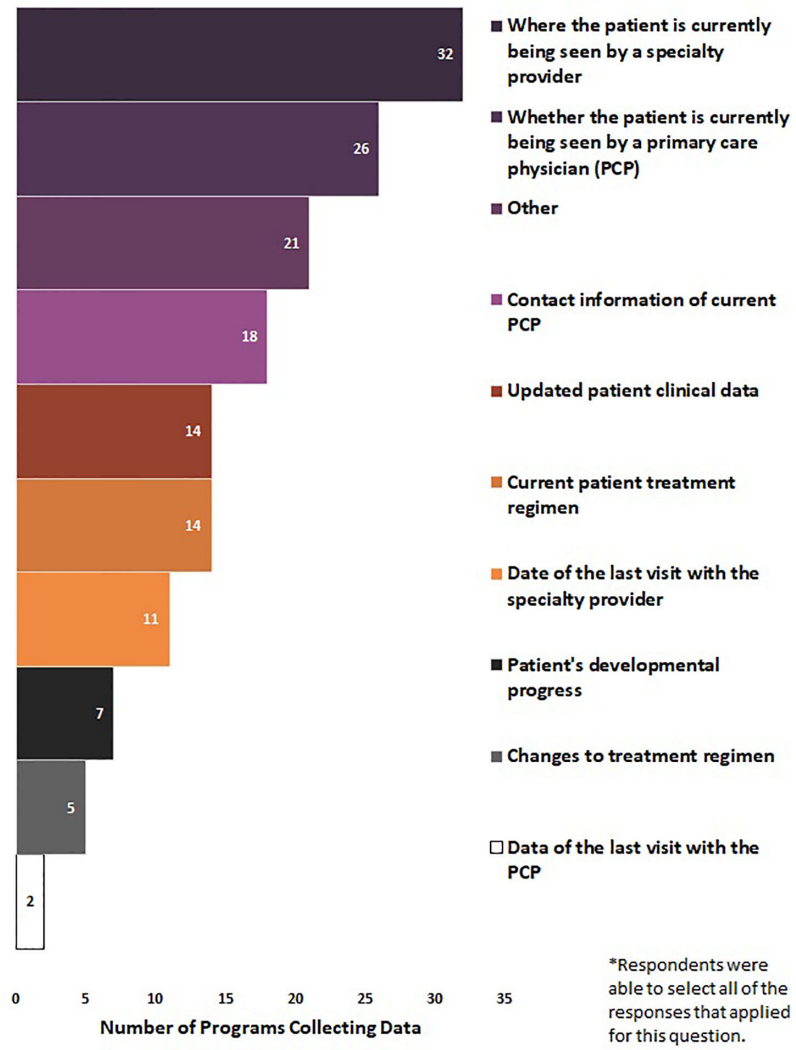

FIGURE 5 | Long-term follow-up data elements collected by newborn screening programs for SCID (January 2020) (N = 41).

To date, each of these screening methods have been successfully implemented; therefore, NBS programs select the platform best suited for their laboratory's conditions (6). An up-to-date list of screening methodologies is maintained in a publicly available Screening Methodologies and Targets Report in the NewSTEPs Data Repository (7).

To maximize the benefits of NBS, key stakeholders must be informed and families must have access to effective counseling and appropriate care over time (13). Currently, wide variation exists in communication pathways for out-of-range SCID NBS. While PCPs, other providers and immunologists are commonly notified by NBS programs of out-of-range SCID screens, less than half of NBS programs reported sharing results with hospitals or other submitters and public health nurses. Hospital staff and public health nurses may not be the medical professionals who directly share results with families; however, these stakeholders may benefit from being informed of out-of-range results. By closing the feedback loop, all stakeholders who are involved in the NBS system can be reminded of the important role they play in the early detection of disorders.

Sixteen percent of NBS programs reported they notified only one stakeholder of an out-of-range newborn screen. These survey results suggest that there are opportunities to expand notification of results to multiple stakeholders and to perform education to ensure patients are successfully connected with ongoing care.

The introduction of a new disorder on an NBS panel requires coordinated efforts to educate providers, parents, and the general public about the disorder. Examples of educational materials for these groups can include webinars, brochures, factsheets, newsletters, videos, presentations, conference exhibits, and organized awareness weeks. Many NBS programs utilize state

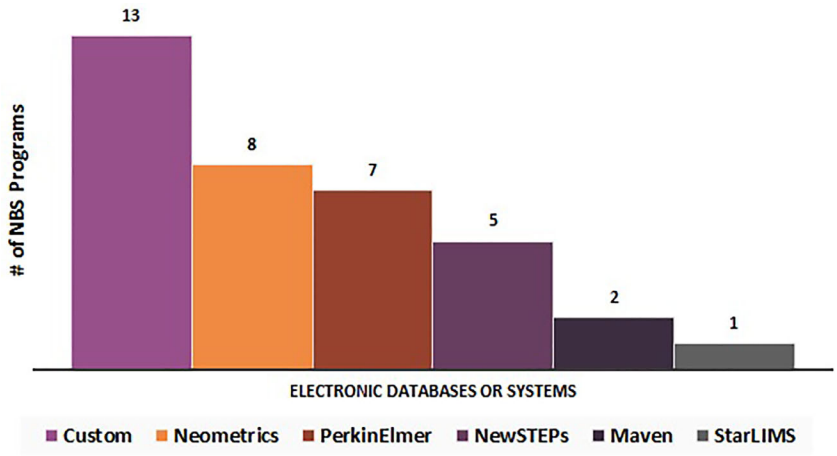

FIGURE 6 | Electronic databases or systems utilized by newborn screening programs for long-term follow-up data elements (N = 33).

websites as a modality for distribution of educational materials and some distribute materials by harnessing partner organizations' networks (6).

The ACHDNC states the principal goal of long-term follow-up is to assure the best possible outcome for individuals with disorders identified through NBS (14). Long-term follow-up allows for a better understanding of the health outcomes of newborns diagnosed with SCID through NBS. This, in turn, allows for continuous quality improvement within the system to identify areas in which additional resources are needed, and to ensure infants identified with SCID by NBS are able to benefit from this early diagnosis throughout their lifespan. Unfortunately, lack of coordinated efforts, along with limited funding, has made it challenging for NBS programs to develop comprehensive long-term follow-up systems (14).

Although confirmed screening positive children are under clinical care, survey results show that less than half of all NBS programs are following patients after they receive a confirmed SCID diagnosis. Despite not having official long-term follow-up systems in place, many NBS programs are collecting data to track the number of patients lost to follow-up, track clinical outcomes of patients, evaluate the performance of specialty providers, as well as to ensure timely follow-up and treatment initiation, to track the number of out-of-range, inconclusive, false positive screens, false negative screens, and associated diagnoses, and to use in broader publication reporting.

This survey reflects a snapshot in time; completed surveys were received between November 2019 and January 2020. Thus, NBS programs who reported "not at this time" to survey questions may have been pursuing activities to get them closer to implementing screening, data exchange, more robust follow-up, strengthening educational programs, and establishing communication pathways. While this survey does reflect nuances that were reported in the "other" category, there may have been NBS programs that chose not provide additional relevant information as well.

No conclusions can be drawn as to which NBS protocols may be considered a best practice in regards to improving outcomes for newborns with out-of-range SCID screening results. Additional studies are needed to evaluate the effectiveness of stakeholder communication, educational outreach, and utility of long-term follow-up data.

CONCLUSION

NBS is a comprehensive system that includes laboratory testing, diagnosis, follow-up, treatment, education and evaluation. To be effective and successful, the NBS system requires a robust laboratory capability to perform early and accurate detection of disorders, adequate resources to perform education and training in NBS, and to refer newborns for treatment upon identification of disorder.

This paper reveals opportunities to enhance the SCID NBS system by expanding communication pathways and educational outreach and establishing more formal long-term follow-up. As SCID NBS programs and treatments evolve, continued knowledge dissemination regarding lessons learned will further enable programs like SCID Compass to enhance linkages between families and services and to further elaborate long-term follow-up strategies for infants identified through NBS (15).

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because access requires permission from state and territorial newborn screening programs. Requests to access the datasets should be directed to ruthanne.sheller@aphl.org.

AUTHOR CONTRIBUTIONS

Conceptualization: RS, SS, JO, EG, SE, CY, and TP, Methodology: RS, SS, JO, and EG. Formal analysis: RS, SS, EG, SE, CY, and TP. Writing: RS and SS. Writing—review and editing: RS, SS, EG, SE, CY, TP, JO, JB, and BF. Visualization: EG. Project administration: AH. Funding acquisition: JO. Supervision: SS and JO. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.577853/full#supplementary-material

REFERENCES

1. Ojodu J, Singh S, Kellar-Guenther Y, Yusuf C, Jones E, Wood T, et al. NewSTEPs: The establishment of a national newborn screening technical assistance resource center. *Int J Neonatal Screen* (2018) 4:1. doi: 10.3390/ijns4010001
2. Watson MS, Mann MY, Lloyd-Puryear MA, Rinaldo P, Howell RR. American College of Medical Genetics Newborn Screening Expert Group. Newborn screening: Towards a uniform screening panel and system. *Pediatrics* (2006) 117:3. doi: 10.1542/peds.2005-2633I
3. Van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal newborn screening for severe combined immunodeficiency (SCID). *Front Pediatr* (2019) 7:37. doi: 10.3389/fped.2019.0037
4. Puck JM. Newborn screening for severe combined immunodeficiency and t-cell lymphopenia. *Immunol Rev* (2019) 287:1. doi: 10.1111/imr.12729
5. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott J, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the united states. *JAMA* (2014) 312:7. doi: 10.1001/jama.2014.9132
6. Association of Public Health Laboratories (APHL). *Expanding the reach of SCID testing: A report on the severe combined immunodeficiency newborn screening implementation experience*. Silver Spring, MD: NewSTEPs (2018). Available at: https://www.newsteps.org/resources/expanding-reach-scid-testing-report-severe-combined-immunodeficiency-newborn-screening.
7. NewSTEPs. *NewSTEPs data repository* (2020). Available at: https://data.newsteps.org/newsteps-web/ (Accessed May 20, 2020).
8. Sontag MK, Sarkar D, Comeau A, Hassell K, Botto LD, Parad R, et al. Case definitions identified by newborn screening publichealth surveillance. *Int J Neonatal Screen* (2018) 4:2. doi: 10.3390/ijns4020016
9. Immune Deficiency Foundation. *Immune deficiency foundation awarded $4 million HRSA grant for national wide screening and education program for severe combined immunodeficiency (SCID)* (2018). Available at: https://primaryimmune.org/news (Accessed May 20, 2020).
10. PerkinElmer. *PerkinElmer announces first commercial availability of newborn screening test for severe combined immunodeficiency (SCID) in the u.s. and canada* (2017). PerkinElmer. Available at: https://ir.perkinelmer.com (Accessed May 20, 2020).
11. Office of Disease Prevention and Health Promotion. *Healthy People 2020*. (2020). Available at: https://www.healthypeople.gov/.
12. 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. doi: 10.1177/00333549101250S211
13. Kavanagh PL, Wang J, Therrell BL, Springz PG, Bauchner H. Communication of positive sickle cell, disease and sickle cell trait: Variation across states. *Am J Med Genet* (2008) 148C:15–22. doi: 10.1002/ajmg.c.30160
14. Kemper A, Boyle C, Aceves J, Doughtery D, Figge J, Fisch JL, et al. Long-term follow-up after diagnosis resulting from newborn screening: Statement of the US Secretary of Health and Human Services' Advisory Committee on Heritable Disorders and Genetic Diseases in Newborns and Children. *Genet Med* (2008) 10:259–61. doi: 10.1097/GIM.0b013e31816b64f9
15. Raspa M, Lynch M, Squiers L, Gwaltney A, Porter K, Peay H, et al. Information and emotional support needs of families whose infant was diagnosed with scid through newborn screening. *Front Immunol* (2020) 11:885. doi: 10.3389/fimmu.2020.00885

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