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Article

Newborn Screening for Primary Immune Deficiencies with a TREC/KREC/ACTB Triplex Assay—A Three-Year Pilot Study in Sweden

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Abstract: *Background*: Screening newborns for severe combined immunodeficiency (SCID) has become essential, since efficient methods to identify infants with these disorders exist and early stem cell transplantation is life-saving. *Method*: We performed a three-year screening trial in Stockholm comprised of 89,462 newborn infants. The number of T-cell receptor excision circle (TREC)/kappa-deleting recombination excision circle (KREC)/β-actin (*ACTB*) copies were quantified simultaneously by real time polymerase chain reaction (PCR) in 3.2 mm punches from dried blood samples taken in the regular neonatal screening program. *Results*: Five patients with immune deficiencies were identified: two with SCID caused by mutations in the Artemis- and adenosine deaminase gene, respectively, one with ataxia telangiectasia and two with reversible agammagloblinemia, which so far, is of unknown cause. This points to an incidence of SCID at the same level as in other studies (around 1:50,000). In 19 recalled infants, low KREC levels and in one case, also low TREC levels, were caused by immunosuppressive treatment of the mother during pregnancy. The levels normalized within a month in all these infants. The total recall rate was 0.10%, and 40% of the recalled infants were born prematurely (<37 weeks gestation). Among 69 patients with inborn errors of metabolism screened retrospectively, only two, who were severely ill with organic acidemias when the sample was taken, and two with mitochondrial disorders, screened positive.

Keywords: severe combined immunodeficiency (SCID); primary immune deficiencies (PID); T-cell receptor excision circle (TREC); kappa-deleting recombination excision circle (KREC); newborn screening; neonatal screening; azathioprine; tacrolimus; Bruton; inborn errors of metabolism

1. Introduction

Newborn screening (NBS) for phenylketonuria (PKU) was initiated in Sweden in 1965, a couple of years after the development of the screening procedure for PKU with blood samples dried on filter paper (DBS) [1]. Since then, galactosemia (1967), congenital hypothyroidism (1980), congenital adrenal hyperplasia (1986), biotinidase deficiency (2002) and expanded screening by tandem mass spectrometry (19 disorders, 2010) have been added to the Swedish NBS program [2]. A centralized procedure for the evaluation of new disorders, led by the Swedish National Board of Health and Welfare, was implemented in 2012. In that procedure, 15 paragraphs were evaluated concerning methodology, ethical considerations and cost benefit, based on the publications by Wilson and Jungner followed by Andermann et al. [3,4] A selected group of different stakeholders would then evaluate and write a recommendation for or against the inclusion of the new disorder and screening for primary immune deficiencies (PID) is now being evaluated [5]. The Swedish neonatal screening is centralized to one laboratory and the annual birth rate is presently 115,000.

Primary immune deficiencies (PID) encompasses a group of more than 250 inherited disorders of immunity, including defects of T-cells, B-cells or a combination of these, as well as complement or granulocyte defects [6]. The incidence of severe PID, in need of immediate treatment, has been estimated to approximately 5–10 per 100,000 newborns. Polymerase chain reaction (PCR)- based quantification of T-cell receptor excision circles (TRECs) extracted from DBS is employed to screen newborn infants for severe combined immune deficiencies (SCIDs) in several states in the USA [7–10].

A similar method for the analysis of kappa-deleting recombination excision circles (KRECs) has been developed for the quantification of novel B-lymphocytes [11]. Patients with severe B-cell deficiency such as X-linked agammaglobulinemia (XLA) can be identified with the KREC assay. A triple assay combining TREC and KREC and β-actin (*ACTB*) as control was developed [12] for neonatal screening. This was employed in a pilot screening of newborn infants for PID, over the course of three years in the County of Stockholm and we have reported the results of the first two years previously [13]. In this paper, we summarize the results from the whole study period.

2. Materials and Methods

2.1. Patients

The NBS pilot study for PID was offered to all newborns born in Stockholm County from 15 November 2013 to 14 November 2016. The parents were informed about the study on two occasions during the pregnancy, and at the time of sampling. For 38 infants, the parents chose not to participate.

In the first two years of the pilot study, samples from 58,834 newborns were analyzed [13]. In the third year, additional samples from 30,628 newborns are included (total 89,462 for the whole three-year study), Figure 1. For 15 infants, a new sample was requested due to inadequate sampling. All results were normal in the new samples. A known source of error for inadequate sampling is the use of blood containing heparin which interferes with DNA-extraction [14,15].

The blood sample for the PID study was collected onto the same Perkin Elmer 226 filter paper and at the same time as the regular NBS sample was taken. In Sweden, NBS samples are taken as soon as possible after 48 h of age, usually at a mean age of 2.8 days. Samples are sent to the laboratory by ordinary mail. The average age at recall because of a positive screening result in the established program is 6 days. For the PID screening, patients with very low TREC and/or KREC levels were recalled immediately, whilst patients with slightly higher levels were recalled after approximately 3 weeks, or even later.

During the study, DBS samples of patients with diagnosed SCID, born either before the pilot-screening or outside the Stockholm County, were also analyzed upon request of referral doctors.

Additionally, a group of 69 patients with different inborn errors of metabolism (IEM) were analyzed, with sample dates ranging from 1984 to 2016. For these, both the screening sample and the DBS sample taken at recall, were analyzed when available.

The regional ethical board in Stockholm approved the studies (Ethical permit 2013/414-31/4, 2013/1834-32 and 96 04 01, D nr 63/96).

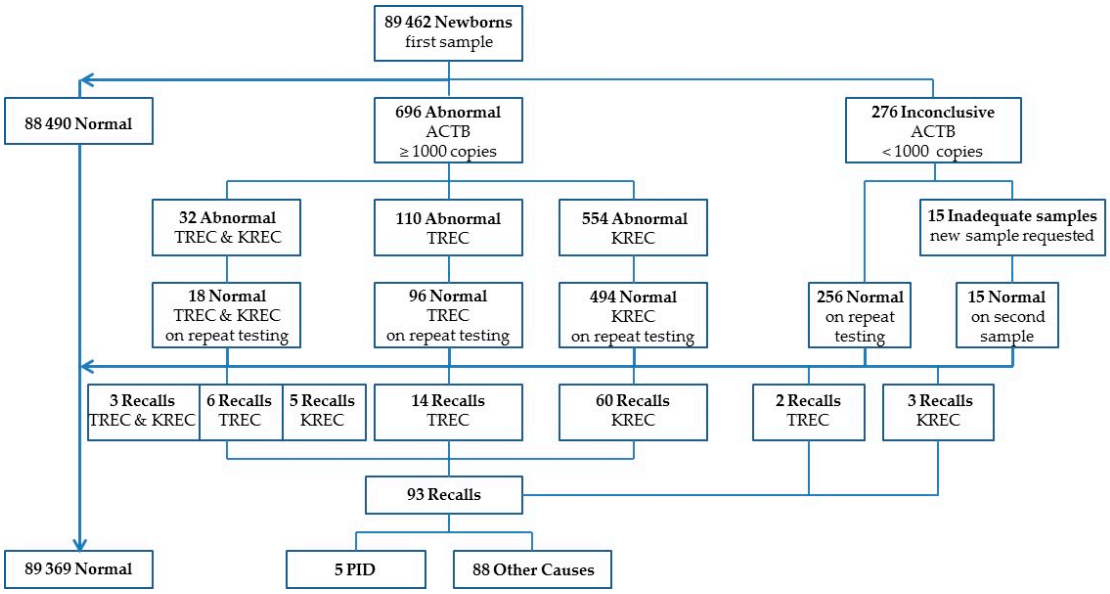

Figure 1. Summary of Newborn screening (NBS) results between 15 November 2013 and 14 November 2016.

2.2. Method

The TREC/KREC assay was performed as described previously, [12,13]. Briefly, DNA was extracted from single 3.2 mm diameter punches of the DBS in 96 well plates. Plates included three control punches: for low TREC, low KREC, low TREC and KREC; as well as two negative controls. Quantitative triplex real time PCR for TREC, KREC and *ACTB* was performed on a Viia7 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). *ACTB* was included as a control of DNA extraction and quality of the assay. Values of *ACTB* were considerad only in case of TREC and/or KREC values below cut-off for repeat test. Two cut-off levels were used—repeat test; 25 and 15 copies per punch for TREC and KREC, respectively and recall, Table 1. Repeat test was performed on the same first screening sample to make sure that families were not disturbed unnecessarily or true positive cases were missed. During the three-year period, the cut-off levels for recalls were adjusted based on the number of recalls, Table 1. All samples with TREC and/or KREC values below repeat test cut-off were reanalyzed in duplicate. A newborn was recalled for clinical evaluation if the mean values of the three analyses of TREC and/or KREC were below recall levels and the simultaneously determined *ACTB* control was above 1000 copies per punch.

The clinical evaluation was performed by a pediatrician who is specialized in PID. A repeat DBS sample was taken along with other hematological investigations as decided by the clinician. For infants born prematurely and still in care at a neonatal unit, the clinical evaluation, along with a repeat DBS sample, was taken care of by a neonatologist.

Inconclusive samples, *ACTB* <1000 copies per punch and KREC and/or TREC copies below repeat test cut-off values, were reanalyzed taking a new punch from another blood spot on the filter paper sample. If still inconclusive, a punch from each blood spot (usually four) on the sample was analyzed. If a sample was still inconclusive after the repeat testing, it was considered inadequate and a new sample was requested.

3. Results

Of the 89,462 samples from newborn infants, 696 samples were below repeat test levels for TREC and/or KREC. These samples were reanalyzed in duplicate and 88 infants were recalled. A total of 276 samples were inconclusive in the first analysis and out of these, five samples were below recall in TREC or KREC after repeat testing and the infants were recalled for clinical evaluation. A second sample was requested from 15 newborns due to inadequate sampling and analyzed with normal results, Figure 1.

Repeat testing was performed on 110 newborns (67 males) with abnormal TREC values in the first analysis. Of these 57 samples were still below rerun cut-off for TREC (25 copies per punch) but above recall level (10 copies per punch) on repeat testing. For samples, abnormal in both TREC and KREC in the first analysis, 14 were still below rerun cut-offs for TREC and KREC (25 and 15 copies per punch, respectively) but above recall values. These samples were considered normal.

The total recall incidence was 1/960 screened infants. Depending on the recall levels in use, the recall incidence varied from 1/470 to 1/2570. Most infants (74%) were recalled due to low KREC levels. No infants, low in KRECs only, were confirmed true positive. Excluding KREC recalls, the recall incidence decreases to 1/3580 screened infants, Table 1.

Table 1. Number of screened infants and recalls, with cut-offs used during different periods.

Period	Screened Infants	Recall Values		Number of Recalls		
		TREC *	KREC *	TREC	KREC	TREC/KREC
1	16,582	15	10	6	29	0
2	28,298	8	4	3	7	1
3	44,582	10	6	13	32	2
Total	89,462	-	-	22 **	68	3 ***

* TREC and KREC is given as copies per 3.2 mm DBS punch; ** Three true positive cases; *** Two true positive cases.

3.1. Recalled Patients

Infants with absent or very low TREC and/or KREC levels were immediately admitted for further investigations with flow cytometry, genetic workup and treatment. For the majority of infants who had low KRECs only, a follow-up sample for TREC and KREC analysis was collected around the age of three weeks, by which time the KRECs had normalized in all but a few cases. In premature inpatient children, a neonatologist was contacted. In the beginning of the study a follow-up DBS sample was taken immediately at recall, and then again after the first year of the study, when the children were older, corresponding to 37 week´s gestational age, or when the child was discharged from the ward if there was no suspicion of an immune deficiency. Except for cases 1 and 3 in Table 2, all prematurely born infants had normalized their TREC and KREC values in the follow-up samples. In case 1 a pediatric immunologist was immediately contacted since the values were so low.

In total, 93 infants were recalled for re-sampling and follow-up due to low TREC and/or KREC levels. Out of these, five patients had a confirmed immune deficiency.

Thirty-seven of the infants were premature, including two who had trisomy 21. Of the 93 children recalled, re-sampling was not available for 6 infants (diseased) and for one the parents declined follow-up. In 19 of the recalls, the mothers had been receiving immunosuppressive therapy. All but one infant was recalled due to low KRECs whilst one was low in both TREC and KREC. The mothers were mostly treated with azathioprine due to inflammatory bowel disease or other autoimmune diseases. Three mothers were also on tacrolimus due to earlier kidney transplantations in two and a uterus transplantation in one. In these 19 immunosuppressed infants, KREC normalized within 3–4 weeks after birth, also in those that were breastfed.

None of all the infants, in whom KREC and TREC levels normalized, have, to date, presented with a history of immune deficiency.

Table 2. True positive patients.

Pat	Gender	Weeks	Grams	TREC *	KREC *	Phenotype	Genetic Diagnosis
1	M	34	1800	0	0	SCID	Artemis deficiency
2	M	39	3110	5	7	CID	Ataxia Telangiectasia
3	M	36	2770	7	205	CID	Unknown
4	M	38	3250	0	0	SCID	ADA deficiency
5	M	40	3445	8	180	CID	Unknown

* TREC and KREC is given as copies per 3.2 mm DBS punch, CID = combined immune deficiency, weeks = gestational week, grams = birth weight.

3.2. True Positive Patients

After confirmatory re-sampling, three infants had low levels of both TREC and KREC, while two had low TREC levels only, Table 2.

These infants (all males) were further investigated for immunodeficiency disorders. The first patient (zero in TREC and KREC) had a homozygous splice-site mutation in Artemis with absent protein expression (*DLCLRE1C*, c.333 + 2T > G). He underwent a successful haploidentical stem cell transplant (HSCT), with the mother as donor, after administering antithymocyte globulin (ATG) as conditioning and α/β-depletion of the graft, at age two months. He had a cytomegalovirus (CMV) infection both before and after the transplant, followed by repeated reactivations. He is now two years and two months, has a complete donor chimerism and is in good health. The subcutaneous IgG treatment that has been administered since the beginning is presently being slowly withdrawn.

The second baby carried compound heterozygous mutations in the ataxia-telangiectasia mutated (*ATM*) gene (c.[3673C>T];[8653_8654insT]) and had a positive chromosomal radio-sensitivity test. He was T-lymphopenic and had hypogammaglobulinemia including IgA deficiency. He was treated with subcutaneous immunoglobulin injections and antibiotics for prolonged viral and bacterial upper respiratory tract infections. He did not have any other clinical features of ataxia-telangiectasia until he past one year of age, when slight neurological symptoms affecting his balance appeared.

The third child, with low TRECs only, was found to have T-cell lymphopenia (TCL) and hypogammaglobulinemia, but whole genome sequencing (WGS) has not, so far, revealed any relevant mutations. He received subcutaneous gammaglobulin in the first year, but has since then had normal immunoglobulin levels. Presently, at age one year and nine months, he has a remaining CD3 lymphopenia (0.9×10^9/L), while mitogen reactivity and antibody production are normal and he is clinically well.

In the fourth patient, who had zero TREC and KREC, an adenosine deaminase (ADA) deficiency SCID was diagnosed (*ADA*, c.[7C>T];[7C>T]), by gene sequencing as well as by assaying a very low erythrocyte ADA level. After initial Peg-ADA enzyme replacement therapy, with increasing T-cell counts, the child underwent a successful HSCT at the age of four months. He was conditioned with ATG, fludarabine plus treosulfan and received stem cells, α/β-depleted, from a haploidentical donor, his father. An acute graft versus host skin reaction and a CMV reactivation ensued. Almost one year later, at age 14 months, he is alive and well and the immunoglobulin and cyclosporine are being slowly tapered.

In the fifth infant, with low TRECs only, TCL and hypogammaglobulinemia were diagnosed, while WGS did not reveal any relevant mutations. He received subcutaneous gammaglobulin but seems to have an emerging autonomous antibody production, allowing tapering of substitution. At 11 months of age he still has a CD3 lymphopenia (0.8×10^9/L), however the mitogen reactivity is normal and he is clinically well.

3.3. Retrospective Analysis of PID Patients

During the time of the study, we had several inquiries from clinicians about the levels of TRECs and KRECs in different patients not born in Stockholm County or born before our study started. These

were all patients in whom PID was either already known or suspected. Patients found to have values below our cut-off levels are listed in Table 3. For patient 1, the whole Bruton agammaglobinemia tyrosine kinase (*BTK*) gene was sequenced without identification of any pathogenic mutations.

Table 3. Patient inquiries.

Pat	Gender	Weeks	Grams	TREC *	KREC *	Phenotype	Genetic Diagnosis
1	M	38	2875	48	0	Agammaglobulinemia	nd
2	F	41	3185	0	0	SCID	Artemis deficiency
3	M	39	3200	0	86	SCID	*IL2RG*
4	M	35	un	7	17	Severe lymphopenia	*UBE2A* **
5	M	41	3500	0	0	SCID	Artemis deficiency
6	F	41	3655	0	0	SCID	*RAG1*

M = male, F = female, un = unknown, nd = not determined; * copies per 3.2 mm DBS punch, ** UBE2A deficiency syndrome with Xq24 deletion, weeks = gestational week, grams = birth weight.

3.4. Retrospective Analysis of TREC and KREC in Patients with Inborn Errors of Metabolism

We have analyzed NBS samples from a selection of 69 patients with different types of inborn errors of metabolism (IEM). The results are summarized in Appendix A, where the patients are divided into groups, depending on the type of IEM. The disease, as well as the gene, if the genetic analysis has been performed, are reported. Neonatal screening for methylmalonic-, propionic-, and isovaleric acidemia has been performed in Sweden since November 2010, explaining why some patients have been detected by clinical presentation.

Eight samples from patients with galactosemia, identified during recent years, were analyzed along with the samples taken at recall of the infants. For mitochondrial disorders, samples from patients with different genetic defects were included and information about frequent infections was reported. Only a few patients have levels below the cut-offs of the PID screening. Within the group who have a defect of the amino acid metabolism, patient 15, with a severe form of propionic acidemia, and who died neonatally despite screening, had a KREC level of only 4 copies per punch. Furthermore, patient 21, who had a severe form of methylmalonyl-CoA mutase deficiency, had a KREC level of 6 copies per punch. Among the patients with a mitochondrial disorder, patient 50 had TREC level of 3 copies per punch, while patient 55 also had a very low TREC level (11 copies per punch), but would not have been recalled using the recall cut-off of TREC $\leq$10 employed during the last period of the study.

4. Discussion

Newborn screening for primary immune deficiencies by TREC analysis is included in almost all programs in the United States, as well as in some other countries [7,9,16–19]. With this methodology, patients with defects in the production of T-cells can be identified, but isolated B-cell defects escape detection. The development of a triplex assay for the simultaneous quantitation of TREC/KREC and *ACTB* has been shown to identify patients who also have isolated B-cell defects like X-linked agammaglobulinemia and Nijmegen breakage syndrome [11,12]. We now report the results of an NBS trial in the County of Stockholm during the years 2013–2016 with the TREC/KREC/*ACTB* triple assay.

A summary of the screening results is presented in Table 1. Among the 89,462 samples analyzed as singletons only, a total of 276 samples had to be retested (0.3%) because of low *ACTB* and KREC and/or TREC levels, indicating a failure of the assay. All except 20 came out normal and among these, a total of five infants were recalled because of low TRECs or KRECs, while a new sample had to be requested from 15 infants (0.017% of the total screened cohort). This is lower than reported from the first three years with TREC screening in Wisconsin (0.14%) [20] and the initial two years in California (0.25%) [9], indicating that the TREC/KREC/*ACTB* triple assay does not give rise to more repeat sampling caused by failures of the analysis than the TREC assay.

We employed retest levels, which were higher than the recall levels, to make sure that we did not miss any infants with low TREC or KREC levels, since the first analysis was as singletons. Another advantage is the possibility to go back and perform record studies of these individuals in the future to find out if they have had signs of an immune deficiency (primary or secondary).

For recalls, we employed different cut-off levels for KREC and TREC during the study period as depicted in Table 1. This was steered by the approximate number of recalls in relation to the birth rate. The recall for the total study was 0.10% and this includes premature infants (gestational age <37 weeks). The corresponding recall rates were 0.07% and 0.09%, respectively, in the Wisconsin and California studies [9,20]. The main part of the recalls was, however, caused by low KREC levels in our study. If only recalls caused by low TRECs are included, the recalls would only have been 0.03% of the screened population. Interestingly, we had a lower fraction of preterm infants among the recalled newborns in Sweden: 37 out of 93 (40%), while the Wisconsin study reported 94 out of 157 (60%). In the California study 106 infants out of 161, who underwent immunophenotyping by flow cytometry, were preterm. Preterm infants, recalled because of TREC levels below our cut-off, amounted to 17 (18% of recalls). We have thus chosen narrower recall levels, to only find patients with SCID, which may miss other forms of primary and secondary immune deficiencies. At the same time, we are not catching as many preterm infants as those programs which have higher recall levels for TREC. We do, so far, not know of any missed cases with PID born in the screened cohort.

Among the recalled infants, two had Down syndrome (0.002%) and were prematurely born. An over-representation of infants with Down syndrome among recalled children without PID has been described earlier when screening with TREC [17]. A total of 19 newborns were recalled because of immunosuppressive therapy of the mother during pregnancy, mainly with azathioprine for inflammatory bowel disease, but with tacrolimus in three mothers who had received kidney transplants (two) and a uterus transplant (one) prior to their pregnancies. All infants had KRECs below cut-off and one also had a TREC level below cut-off. All normalized within 3–4 weeks after birth. Low TREC levels, because of immunosuppressive treatment, has been described before [21] but the B-cell synthesis is obviously more sensitive to these treatments, as reported earlier [13,19]. The future will tell if these infants will have any problems caused by this immune depression during fetal life.

None of the recalled infants without proven immune deficiency have turned up with any other diseases connected to immune deficiency and they had normal TRECs and KRECs at follow-up, except for those who died prior to re-sampling (six patients) or declined follow-up (one infant). Given our comparably low recall rate, one could speculate that some of the recalled infants will turn out to have some immunological weakness, since this has been found in higher frequencies in other studies [17].

Five patients had immune deficiencies, Table 2. Patient 1 and 4 have SCID (one Artemis and the other ADA deficiency) and are transplanted and doing well. This gives an incidence of 1:45,000 newborns, in line with earlier observations [9,17,20] although our numbers are small and uncertain. Patient 2 with XLA is slowly deteriorating neurologically, while patients 3 and 5 with initial hypogammaglobulinemia are improving and treatment is slowly being withdrawn. A genetic cause has not been found, so far, in these two patients. Interestingly, the two SCID patients had zero copies of TREC as well as KREC, while the other three patients had TREC levels closer to our cut-off. The two patients with initial agammaglubulinemia of unknown cause had almost normal levels of KRECs, which is puzzling. Hopefully the cause of their initial hypogammaglobulinemia will be clarified.

Among samples retrieved from patients born outside our study, but suspected of having an immune defect, a total of eight had a verified defect, Table 3. Of these, six had SCID and they all had zero copies of TREC per punch, while KREC was low but detectable in two patients with SCID of unknown cause and one patient with IL2RG. Patient 1 with agammaglobulinemia had zero KRECs but TREC levels above our cut-off and should thus not have been detected by TREC screening only. The genetic cause of the agammaglobulinemia is not yet clear. The *XLA*-gene has been investigated without finding any mutation. Whole genome sequencing will be performed in search of the genetic cause.

It is known that IEM can cause a secondary immunodeficiency [22]. In the first publication, demonstrating the value of the TREC assay in NBS, IEMs have also been reported to cause low TREC levels [20]. We evaluated a group of patients with IEM with our triplex assay.

Of the investigated 69 patients with an IEM, only three had values below the cut-offs of 10 and 6 for TREC and KREC, respectively, the cut-offs used during the last screening period, Table 1. Patients with propionic acidemia, methylmalonic acidemia (including mutase as well as cobalamin deficiencies) and isovaleric acidemia have been shown to suffer from depression of lymphocyte production [23]. Among the 24 patients with one of these disorders, only two were positive. Both had KREC levels below our cut-off and had neonatal onset of severe symptoms. One had propionic acidemia and expired in the neonatal period, whilst the other one had metylmalonic acidemia caused by a total mutase deficiency and is alive. This is not surprising, since the depression of lymphocyte production occurs when toxic metabolites accumulate during metabolic decompensation and accumulation of toxic metabolites [23]. The screening sample is usually taken before this occurs, resulting in normal TREC/KREC levels in most patients.

Other IEMs may present primarily with an immunologic phenotype, for example, lysinuric protein intolerance [22]. The two patients we analyzed had normal TREC or KREC levels. Patients with disorders of cobalamin transport are also known to show a SCID phenotype [22], but this was not the case in the patient with a transcobalamin II defect investigated here.

We analyzed the screening sample from eight patients with galactosemia and all had normal levels of TRECs and KRECs, also on the recall sample. Infants with galactosemia are sensitive to infection with bacteria, especially *Escherichia Coli*, starting when high levels of galactose and galactose-1-phosphate have accumulated during the second week of life [24]. The underlying mechanism is not clear and it has not been shown to be caused by lymphopenia. It is thus not surprising that all these patients had normal screening results.

We selected several patients with mitochondrial diseases, some of whom have had repeated infections since early ages, indicating a functional immune deficiency. However, only one patient with Leigh syndrome due to a *SERAC1* mutation showed a low TREC level. None of the other mitochondrial patients had levels below cut-off in the neonatal screening sample. Included were patients with a *POLG* mutation, as well as Pearson syndrome, for which neutropenia and bone marrow failure have been reported [25,26].

To summarize, we screened almost 90,000 infants for PID, using a TREC/KREC/*ACTB* triplex assay and identified two children with SCID, one with XLA and two with neonatal hypogammaglobulinemia, which so far, is of unknown cause. The recall rate has been 0.10% and the PPV 5.5%.

We have not identified any patient with PID based exclusively on low KREC levels in the screening sample during the three-year screening period. On the other hand, we have shown earlier that KREC is essential for the identification of infants with Nijmegen breakage syndrome and X-linked agammaglobulinemia in DBS screening samples [12]. KREC determination adds support to the SCID diagnosis (Table 2), since KREC as well as TREC were zero in the patients with SCID. The inclusion of KREC in the screening program has resulted in the main part of the false positive outcomes, but it is still acceptably low. We thus conclude that the triple assay is the most suitable for the neonatal screening for SCID.

There is an urgent need for screening of PID as it saves lives of patients with SCID by enabling stem cell transplantation before they have received life threatening opportunistic infection. Another advantage with early diagnosis of PID is avoidance of severe infections caused by viable vaccines.

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Author Contributions: Lennart Hammarström initiated the study. Stephan Borte developed the assay. Michela Barbaro, Annika Ohlsson, Susanne Jonsson, Rolf H Zetterström and Ulrika Von Döbeln performed the screening. Term children with a suspected PID were referred to Jacek Winiarski. for evaluation and follow-up. All authors contributed to the final text.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix

Table A1. Summary of results for patients with inborn errors of metabolism.

Pat	Disorder	Gene [1]	Screening Detected	Comments	TREC Copies	KREC Copies	TREC Recall	KREC Recall
Cobalamin and Folate Transport and Metabolism Disorders								
1	Methylmalonic acidemia, CblA	*MMAA*	no	Onset at ten months	184	196		
2	Methylmalonic acidemia, CblA	*MMAA*	yes		179	56		
3	Methylmalonic acidemia, ClbB	*MMAB*	no	Neonatal onset	300	55		
4	Methylmalonic acidemia and homocystinuria, CblC	*MMACHC*	no		167	129		
5	Methylmalonic acidemia and homocystinuria, CblC	*MMACHC*	no	Onset at three months	64	27		
6	Methylmalonic acidemia and homocystinuria, CblC	*MMACHC*	yes		61	100	179	181
7	Methylmalonic acidemia and homocystinuria, CblC	*MMACHC*	yes		188	105		
8	Homocystinuria-megaloblastic anemia, CblG	nd	no	Died at ten months	33	181		
9	Homocystinuria-megaloblastic anemia, CblG	nd	no	Neonatal onset	76	118		
10	Homocystinuria-megaloblastic anemia, CblG	nd	no		82	100		
11	Transcobalamin II deficiency	nd	no		132	147		
12	Homocystinuria due to MTHFR deficiency	*MTHFR*			283	60		
Amino Acid Metabolism								
13	Propionic acidemia	*PCCB*	no		59	47		
14	Propionic acidemia	*PCCB*	no	Symptoms at day two	62	50		
15	Propionic acidemia	*PCCA*	yes	Died neonatally	51	4		
16	Propionic acidemia	*PCCB*	yes	No symptoms	98	157	236	281
17	Propionic acidemia	*PCCB*	yes	Mild form	269	51		
18	Propionic acidemia	nd	yes		59	11	34	25
19	Propionic acidemia	*PCCA*	yes		220	190	267	91
20	Methylmalonic acidemia due to methylmalonyl-CoA mutase deficiency	*MUT*	no		73	26		
21	Methylmalonic acidemia due to methylmalonyl-CoA mutase deficiency	*MUT*	no	Neonatal onset	44	6		
22	Methylmalonic acidemia due to methylmalonyl-CoA mutase deficiency	*MUT*	no	Neonatal onset	102	42		
23	Methylmalonic acidemia due to methylmalonyl-CoA mutase deficiency	*MUT*	no	Onset at nine months	75	68		
24	Isovaleric acidemia	nd	no		197	53		
25	Isovaleric acidemia	nd	no					
26	Isovaleric acidemia	*IVD*	yes	No symptoms	205	151	187	132
27	Isovaleric acidemia	*IVD*	yes		187	150	217	145
28	Isovaleric acidemia	*IVD*	yes		240	55	431	109
29	Isovaleric acidemia	*IVD*	yes		196	103	127	61
30	Lysinuric protein intolerance	nd	no		81	92		
31	Lysinuric protein intolerance	nd	no		36	289		

Table A1. *Cont.*

Pat	Disorder	Gene [1]	Screening Detected	Comments	TREC Copies	KREC Copies	TREC Recall	KREC Recall
Mitochondrial Disorders								
32	Pearsons syndrome	mtDNA deletion	no	Anemia	58	50		
33	Pearsons syndrome	mtDNA deletion	no	Anemia	132	148		
34	Pearsons syndrome	mtDNA deletion	no		28	12		
35	Leigh syndrome	*MTND1*	no	Anemia	20	14		
36	Leigh syndrome	*MTND3*	no		172	163		
37	Leigh syndrome	*MTND3*	no		113	93		
38	Leigh syndrome	*MTND3*	no		70	75		
39	Leigh syndrome	*MTND5*	no	Frequent infections	72	77		
40	Leigh syndrome	*MTND6*	no		226	78		
41	Leigh syndrome	*MTATP6*	no		100	220		
42	Leigh syndrome	*MTATP6*	no		89	107		
43	Leigh syndrome	*NDUFAF2*	no		265	121		
44	Alpers syndrome	*POLG*	no	Frequent infections	208	108		
45	Alpers syndrome	*POLG*	no		215	238		
46	Alpers syndrome	*POLG*	no	Frequent infections., low IgG, IgA, IgM	85	16		
47	mtDNA depletion syndrome	*TK2*	no		105	33		
48	mtDNA depletion syndrome	*MPV17*	no		119	108		
49	Mitochondrial encephalopathy	*COX10*	no	Anemia, multi organ involvment	24	87		
50	Leigh syndrome	*SERAC1*	no	Frequent infections	3	285		
51	Leigh syndrome	*SERAC1*	no		110	105		
52*	Mitochondrial encephalopathy	*SERAC1*	no		112	161		
53*	Mitochondrial encephalopathy	*SERAC1*	no		70	60		
54	Leigh syndrome	*SERAC1*	no		191	61		
55	Mitochondrial encephalomyopathy	nd	no		11	91		
Galactosemia								
56	Galactosemia	*GALT*	yes		102	61	139	133
57	Galactosemia	*GALT*	yes		158	100	182	172
58	Galactosemia	*GALT*	yes		208	76	110	49
59	Galactosemia	*GALT*	yes		56	128	119	197
60	Galactosemia	*GALT*	yes		163	89	228	129
61	Galactosemia	*GALT*	yes		240	104	155	113
62	Galactosemia	*GALT*	yes		412	45		
63	Galactosemia	*GALT*	yes		169	115	321	261
Disorders of Nucleic Acid Metabolism								
64	Arts syndrome	*PRPS1*	no		93	51		
Congenital Disorders of Glycosylation								
65	Congenital disorder of glycosylation, type Ia	*ALG13*	no		330	118		
Lysosomal Storage Disorders								
66	Gaucher disease	*GBA*	no		123	424		
67	Gaucher disease	*GBA*	no		302	99		
68	Gaucher disease	*GBA*	no		229	113		
69	Aspartyl glucosaminuria	*AGA*	no		246	64		

[1] Genes are indicated if the diagnosis has been genetically confirmed; * siblings; nd = not determined.

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