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ABSTRACT

Primary immunodeficiencies (PID) are congenital disorders of immune competence, which are mainly characterized by a pathological susceptibility to infection. This is often accompanied by severe recurrent infections with drug-resistant, long progressions. In addition, there are associated immune regulation disorders, which may manifest themselves in granuloma formation, auto-immunity, recurrent fever, eczema, lymphoproliferation and chronic intestinal inflammation. More than 240 disease entities have been defined so far as PID, and just as extensive is the spectrum of clinical severity. While the most common congenital immunodeficiencies, such as selective IgA deficiency or C2 complement deficiency, have a mild phenotype which often remains undetected, severe PID are characterized by significant mortality in the first years of life, as well as a serious morbidity with irreversible organ damage. This applies in particular to PID that are defined by the absence or functional anergy of T-lymphocytes (severe combined immunodeficiency; SCID) or B-lymphocytes (e.g. X-linked agammaglobulinemia; XLA). Patients with such severe congenital immunodeficiencies appear to be in perfect health at birth, yet show initial manifestations of SCID between the 14th day and the 4th month following birth; in patients with XLA usually between the 8th and 16th month after birth.

Albeit increasingly becoming appreciated as a relevant health problem, there is a lack of diagnostic procedures and screening profiles that would allow earliest possible diagnosis of patients with severe PID on a population scale. As a superior prognosis could be given upon prompt diagnosis and immediate adequate treatment, one strategy to improve the outcome of severe PID shall be to test newborns for the presence of T and B cells. With the aim to develop a simple and reliable test for newborn screening using the established dried blood sampling system (Guthrie cards), a multiplex real-time quantitative qPCR assay for the quantitation of T cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) as surrogate markers of T and B cell development was designed and evaluated (publication I). This assay was further extended to allow detection of newborns with an inversion of the *UNC13D* gene, causing a severe PID characterized as familial hemophagocytic lymphohistiocytosis (publication III). Furthermore, the feasibility to identify several other severe PID, characterized by a functional defect of T- and B-cell interation (combined immunodeficiency diseases), was assessed using IgA-protein detection in neonatal Guthrie cards. However, this assay provided evidence of a maternal transfer mechanism for IgA, thereby preventing the use of this assay as a screening tool for severe PID (publication II). Finally, the TREC-KREC newborn screening assay was further improved in terms of assay performance and evaluated in retrospective cohorts of patients with the DiGeorge syndrome, or Down syndrome (publications IV and V). In addition, novel second-tier assays for comfirmation of the 22q11 microdeletion or the chromosome 21 triplication have been designed and succesfully tested with neonatal samples.

In summary, new assays and concepts for newborn screening of severe primary immunodeficeincies were designed and benchmarked in retrospective and prospective neonatal dried blood spot samples, thereby underlining the potential of this preventive health care strategy.

LIST OF INCLUDED PUBLICATIONS

I. **Borte S**, von Döbeln U, Fasth A, Wang N, Janzi M, Winiarski J, Sack U, Pan-Hammarström Q, Borte M, Hammarström L: **Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR.** *Blood.* 2012; 119 : 2552-2555.

II. **Borte S**, Janzi M, Pan-Hammarström Q, von Döbeln U, Nordvall L, Winiarski J, Fasth A, Hammarström L: **Placental transfer of maternally-derived IgA precludes the use of guthrie card eluates as a screening tool for primary immunodeficiency diseases.** *PLoS One.* 2012; 7 : e43419.

III. **Borte S**, Meeths M, Liebscher I, Krist K, Nordenskjöld M, Hammarström L, von Döbeln U, Henter JI, Bryceson YT: **Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies.** *J Allergy Clin Immunol.* 2014; 134 : 226-228.

IV. Verstegen RH, **Borte S**, Bok LA, van Zwieten PH, von Döbeln U, Hammarström L, de Vries E: **Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases.** *J Allergy Clin Immunol.* 2014; 133 : 1208-1211.

V. Lingman Framme J, **Borte S**, von Döbeln U, Hammarström L, Oskarsdóttir S: **Retrospective analysis of TREC based newborn screening results and clinical phenotypes in infants with the 22q11 deletion syndrome.** *J Clin Immunol.* 2014; 34 : 514-519.

LIST OF OTHER RELEVANT PUBLICATIONS

I. **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 : 118-130.

II. Speckmann C, Neumann C, **Borte S**, la Marca G, Sass JO, Wiech E, Fisch P, Schwarz K, Buchholz B, Schlesier M, Felgentreff K, Grimbacher B, Santisteban I, Bali P, Hershfield MS, Ehl S: **Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis.** *J Allergy Clin Immunol.* 2012; 130 : 991-994.

III. **Borte S,** von Döbeln U, Hammarström L: **Guidelines for newborn screening of primary immunodeficiency diseases.** *Curr Opin Hematol.* 2013; 20 : 48-54.

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VI. la Marca G, Canessa C, Giocaliere E, Romano F, Malvagia S, Funghini S, Moriondo M, Valleriani C, Lippi F, Ombrone D, Della Bona ML, Speckmann C, **Borte S**, Brodszki N, Gennery AR, Weinacht K, Celmeli F, Pagel J, de Martino M, Guerrini R, Wittkowski H, Santisteban I, Bali P, Ikincioglullari A, Hershfield M, Notarangelo LD, Resti M, Azzari C: **Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots.** *J Allergy Clin Immunol.* 2014; 134 : 155-159.

VII. Sack U, BoldtA, Borte M, **Borte S**: **Novel diagnostic options for immunodeficiencies.** *Clin Biochem.* 2014; 47 : 724-725.

✓III. **Borte S**, Borte M, Pöge A, Sack U: **Identification of severe primary immunodeficiencies in newborns: diagnostic strategies for screening, tracking and follow-up.** *LaboratoriumsMedizin - Journal of Laboratory Medicine.* 2014; 32 : in press.

IX. **Borte S**, Fasth A, von Döbeln U, Winiarski J, Hammarström L: **Newborn screening for severe T and B cell lymphopenia identifies a fraction of patients with Wiskott-Aldrich syndrome.** *Clin Immunol.* 2014; in press.

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LIST OF ABBREVIATIONS

ACTB	Beta-actin; *ACTB* gene
ALC	Absolute lymphocyte count
AT	Ataxia telangiectasia
CBC	Complete blood count
CGD	Chronic granulomatous disease
CID	Combined immunodeficiency
DBS	Dried blood spot(s)
FACS	Flow-assisted cell sorting
FHL	Familial hemophagocytic lymphohistiocytosis
GT	Gene therapy
HIGM	Hyper-IgM-syndrome
HSCT	Hematopoietic stem cell transplantation
HTA	Health technology assessment
IgA	Immunoglobulin A
IgAD	Selective IgA deficiency
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KREC	Kappa-deleting recombination excision circle
NBS	Newborn screening
PCID	Profound combined immunodeficiency
PID	Primary immunodeficiency disease(s)

RTE	Recent thymic emigrants
SCID	Severe combined immunodeficiency
TRAC	T cell receptor alpha constant region
TREC	T-cell receptor excision circle
WHO	World health organization
XLA	X-linked agammaglobulinemia; Bruton`s disease

Foreword

This thesis comprises diagnostic assays and studies applied to the identification of primary immunodeficiency diseases (PID) at the neonatal stage. While the concept of newborn screening is not new, its broader application to severe PID has only come into action in recent years. Originating from pilot studies initiated in the United States in 2008, more than 3 million newborns have been screened so far for severe combined immunodeficiency (SCID), resembling one of the diseases at the severe end of PID in terms of disease-related morbidity and mortality.

My intention, when starting to conduct and organize research in the field of neonatal testing for PID, was to extend the concept of SCID screening to other relevant diseases such as inborn agammaglobulinemias, hemophagocytic lymphohistiocytosis, and eventually defects of phagocytic cell function. Furthermore, my aim was to evaluate newly developed multiplex assays in retrospective and prospective cohorts to support the broader implementation of newborn screening tests for PID. It is rewarding to see what has been achieved during the past few years for the sake of our patients.

Leipzig, August 10, 2014

Stephan Borte

INTRODUCTION

1.1 Clinical concepts of immunodeficiencies

A compromised or abolished function to fight infectious diseases is a major hallmark of immune deficiencies, most commonly resulting from an acquired condition such as iatrogenic immunosuppression (e.g. chemotherapy, radiation, glucocorticoids and anti-rheumatic drugs), malnutrition, aging, malignancies or metabolic diseases, as well as referred by the pathomechanism of chronic infections. In clinical practice, the cause and effect of acquired immunodeficiencies often is well-documented, yet sometimes of complex nature. Besides, the clinical description of the functional relevance of an acquired immune defect is mostly driven by the choice of therapeutic options available, such as antimicrobial treatment in asplenia, immunoglobulin replacement therapy for humoral deficiencies, hematopoietic growth factor supplementation in neutropenia, and hematopoietic stem cell transplantation (HSCT) or gene therapy (GT) for hematopoietic lineage defects.

Primary immunodeficiencies, however, are inborn defects of the immune response and closely relate to our understanding of basic to advanced concepts of immunobiology. As immunology is a relatively new science, it is intrinsically tied to the mechanisms of host and microbe interaction and response, described first in discoveries of Edward Jenner, Robert Koch, Louis Pasteur, Emil von Behring, Shibasaburo Kitasato and Elie Metchnikoff. This mechanistic view has provided the framework for researchers and doctors to classify PID according to the component of the immune system that predominantly is associated with the observed course and phenotype of infectious complications: innate versus

adaptive immune defects; including B cell-, T cell-, and NK-cell disorders, as well as granulocyte defects and complement deficiencies. Furthermore, the networked interaction of immune cells is represented in definitions such as ‘combined immunodeficiencies’, with two or more immune components being affected in function or number, or ‘disorders of DNA repair, somatic hypermutation and class switching’ for molecular defects that affect several cell types. However, definitions and diagnostic criteria for PID are fluidic and subject to ongoing refinement (1-3).

1.2 Primary immunodeficiency diseases (PID)

Genetically determined disorders of protective immune function are commonly referred to be primary immunodeficiency diseases and have been recognized clinically already 60 years ago with the identification of X-linked agammaglobulinemia. Today, the group of PID involves over 200 distinct entities which are scattered to present as phagocytic disorders, complement deficiencies, T-cell deficiencies and - predominantly - antibody disorders (1).

In his enigmatic case report published 1952 in Pediatrics, Ogden Carr Bruton depicted the clinical course, diagnostic workup and therapeutic approach of an 8-year old boy with absent serum immunoglobulins and severe infectious complications, including several episodes of pneumonia, recurrent otitis media and osteomyelitis (4). The case procedures already anticipated the patient-centered approach that is taken nowadays to identify primary immunodeficiency diseases based on warning signs circularization and clinical pattern education in order to alleviate or prevent the impact of a delayed diagnosis. In addition, sophisticated diagnostic tools such as molecular assays suitable for high-

throughput screening of newborns have found their way to the PID field and are currently undergoing evaluation if this step of preventive health care balances the total expenditures for such screening programs compared to the efficiency to diagnose, treat and improve the long-term outcome of PID patients (5). Finally, a modern management of patients requiring substitution of immunoglobulin products, including those diagnosed with (severe) combined immunodeficiencies, primary B-cell immunodeficiencies and selected disorders such as the Wiskott-Aldrich syndrome, has advanced to provide a more sustainable therapeutic effect and to improve the patients` quality-of-life than merely replacing antibody protection (6,7).

Despite these achievements in the field, patients with primary antibody deficiencies in particular are still prone to a significant diagnostic delay, thereby promoting an increased likelihood to develop and progress infection-associated organ damage (8,9). The chronic lung disease, characterized by moderate to severe bronchiectasis observed in these patients, remains an unresolved threat to the overall life expectancy despite virtually normal levels of serum immunoglobulin G (IgG) following replacement therapy (10,11). The clinical experience gathered throughout the years endorses disease awareness and should guide research into newborn screening, disease progression and future therapeutic strategies (12).

Primary immunodeficiencies are not rare diseases and should be considered in all patients with severe or recurrent infections admitted to health care centers. The summarized prevalence ranges from 1 in 500 to 1 in 2.000 of the general population, yet PID are misdiagnosed in many cases because of their prolonged course of disease which often worsens the outcome (8). However, the estimated incidence of severe PID, which is those that would require immediate treatment,

ranges from 2 - 8 per 100.000 live births, making high demands on the effectiveness and availability of diagnostic tests to detect them as soon as possible. The European Society for Immunodeficiencies (ESID) has enrolled more than 15.000 PID patients into a database, allowing deciphering the diagnostic delay and factors influencing prognosis and quality-of-life (13). The registry data indicates that PID associated with acute serious infections, such as severe combined immunodeficiencies (SCID), had the shortest median delays until diagnosis (0.1 – 0.2 years). In contrast, patients with antibody deficiencies were prone to a very long diagnostic delay (4 - 10 years), presumably as a result of the less-life-threatening infections dominating the clinical presentation, paired with a lack of awareness towards PID among the medical professionals (8). In order to reduce the significant morbidity related to antibody deficiency diseases and to improve patients` quality-of-live and the overall survival, critical importance has to be attached to strategies that would allow earliest-possible diagnosis of these patients, e.g. by means of newborn screening, and prompt initiation of immunoglobulin replacement therapy as well as further supportive care.

1.3 Neonatal testing and newborn screening

The purpose of neonatal testing is the early recognition of treatable, mostly genetically-determined diseases that manifest with a high rate of morbidity and mortality. Mass screening of newborn infants started in the early 1960s based on a method developed by Robert Guthrie and Ada Susie for the screening of phenylketonuria (14,15). Peripheral blood from a heel stick was blotted on filter paper (later on often referred as ‘Guthrie card’) at two to five days after birth, dried and sent by postal mail to centralized laboratories for analysis. Once this

sampling procedure was established, new disorders were considered for NBS and it became necessary to create procedures for decision-making, to help deciding which diseases were suitable for a population-based screening. A conference arranged by the World Health Organization (WHO), originally intended to discuss screening for cancer in adults, resulted in a paper written by James Wilson and Gunnar Jungner describing ten criteria, to be considered when a new disorder is proposed for mass screening (16).

The addition of new diseases in neonatal screening programs has been critically determined by the development of methods suitable for the analysis of large numbers of samples up until the mid-1990s. The technological progress since then, allowing simultaneous determination ('multiplexing') of several hundreds of metabolites in dried blood spot samples (DBS) from filter paper, together with novel techniques for the analysis of nucleic acids (DNA and RNA), revolutionized newborn screening. However, with these breakthrough technologies being broadly available, the choice of disorders to be included in NBS has to be carefully scrutinized with respect to cost benefit considerations, the patient perspectives and social acceptance. The complex of problems associated with this process is illustrated by differing decisions made in different countries (14).

In the USA, the congress decided in 2003 to include 29 core and 25 secondary disorders in the NBS program, adding up to a total of 54 inborn diseases that are screened for in newborns. This decision was motivated by thorough investigations, resulting in a priority list of disorders suitable for neonatal screening (17). In recent years, more disorders have been included in this list of recommended diseases to be screened all over the US – one of them being SCID (18). At the other extreme, a health technology assessment (HTA) performed in

Great Britain in 2004 came to the conclusion that a substantial benefit by neonatal screening, based on a multiplexing technology, could only be shown for two disorders (19). Thus, only five disorders are currently included in the general newborn screening program in Great Britain. A recent investigation of the various approaches towards NBS evaluation in different European countries illustrates the considerable heterogeneity of the included disorders, as well as the diversity of the organizational structure of the NBS programs (20).

Newborn screening for SCID as a representative of a severe PID has already been proposed in the late 1990s by Jennifer Puck and Rebecca Buckley, suggesting that a complete (differential) blood count (CBC) might help disclose SCID newborns based on a reduced absolute lymphocyte count (ALC) per milliliter of blood. As this method would have to rely on fresh blood samples from every newborn tested, the efforts for sample logistics and handling would prevent a large-scale implementation. In addition, T-B+NK+ SCID genotypes with elevated numbers of B- or NK-cells might return false-negative test results. Later on, ELISA-like immunoassays for Interleukin-7 were proposed for SCID screening, yet technical issues of inferior sample stability and tedious protein purification prevented broader evaluation (21). Similarly, bead-capture assays for the T-cell receptor-CD3 complex and CD45 were developed, but false-negative results in SCID patients with maternal engraftment or expansion of oligoclonal T cells have been reported, thereby disqualifying this technique from implementation (22). As the availability of a suitable test or examination, that is acceptable to the general population, is a critical prerequisite for evaluation of proposed diseases to be included in NBS programs, it took until 2009 when Mei Baker and Jack Routes returned solid proof for the suitability of the T-cell receptor excision circle (TREC) assay for SCID screening (23).

1.4 Guidelines for newborn screening of severe PID

Severe PID represent a small fraction of all primary immunodeficiency diseases grouped by the clinical need for early and curative or lifelong therapeutic intervention to prevent progressive morbidity or, ultimately, early mortality. Thus, from a clinical point of view, one can imagine a number of severe PID to be evaluated for the development of screening tests and for subsequent inclusion in newborn screening programs. These include, but are not limited to:

- Deficiency of absolute numbers of T cells; i.e. in SCID or complete DiGeorge syndrome
- Defects of the function of T and B cells; i.e. in profound combined immunodeficiencies such as MHC defects and IKK2 deficiency
- Deficiency of absolute numbers of B cells; i.e. in XLA
- Deficiency of absolute numbers of phagocytes; i.e. in Kostmann syndrome
- Defects of neutrophil function; i.e. in chronic granulomatous disease
- Deficiency of NK cell function; i.e. in FHL
- Congenital thrombocytopenias; i.e. in Wiskott-Aldrich syndrome

Even given that different concepts for NBS evaluation exist, most countries warrant compliance of the proposed diseases with the original Wilson-Jungner criteria for mass screening (14), consisting of the following ten items:

1. The condition sought should be an important health problem.
2. There should be an accepted treatment for patients with recognized disease.

3. Facilities for diagnosis and treatment should be available.

4. There should be a recognizable latent or early symptomatic stage.

5. There should be a suitable test or examination.

6. The test should be acceptable to the population.

7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.

8. There should be an agreed policy on whom to treat.

9. The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditure on medical healthcare as a whole.

10. Case finding should be a continuing process and not a "once and for all" project.

As the framework of the Wilson-Jungner criteria was not strictly designed to fit the demands of a neonatal screening evaluation, several countries have made more specific amendments (14,20). Moreover, to improve the ability of decision makers and public health practitioners to stratify disease-specific aspects in the context of a newborn screening setting, it was suggested to expand the Wilson - Jungner framework by supplemental items (24,25):

11. There should be scientific evidence of screening program effectiveness and the benefits of screening should be shown to outweigh the harm.

12. The test may be multiplexed or overlaid onto an existing structure or system.

13. The “diagnostic odyssey” for the patient/family may be reduced or eliminated.

14. Adverse outcome(s) are rare with a false-positive test.

15. Treatment costs may be covered by third parties (either private or public).

16. Testing may be declined by parents/guardians.

17. Adequate pretesting information or counselling is available to parents/guardians.

18. Screening in the newborn period is critical for prompt diagnosis and treatment.

19. Public health infrastructure is in place to support all phases of the testing, diagnosis, and interventions.

20. If carriers are identified, genetic counselling is provided.

21. Treatment risks and the impact of a false-positive test are explained to parents/guardians.

22. The limitations of screening and risks of a false-negative test are explained to parents/guardians.

To demonstrate the practical guidance that is given by the extended Wilson-Jungner framework, a brief evaluation of two severe PIDs currently not included in routine newborn screening programs is shown in Table 1.

		Agammaglobulinemias (XLA)		Familial hemophagocytic lymphohistiocytosis (FHL3)	
	Criteria advocating newborn screening	Scientific evidence available	Uncertain or inconclusive	Scientific evidence available	Uncertain or inconclusive
1	Important health problem	X (26)		X (27)	
2	Accepted treatment	X (28)		X (27)	
3	Facilities for diagnosis and treatment exist	X (29)		X (29)	
4	Recognisable latent stage	X (Publication 1)			X
5	Suitable test system available	X (Publication 1)		X (Publication 3)	
6	Test acceptable to the population		X		X
7	Natural disease history understood	X (26)		X (27)	
8	Agreed policy on whom to treat	X (26)			X
9	Cost of case finding is economically balanced		X		X
10	Case finding is a continuous process	X (26)		X (27)	
11	Scientific evidence for screening efficacy		X		X
12	Test can be overlaid with existing systems	X (Publication 1)			X
13	Reduced diagnostic odyssey	X (26)		X (27)	
14	Adverse outcome rare in false-positives		X		X
15	Treatment costs covered	X		X	
16	Testing may be declined	X		X	
17	Pretesting information is available	X		X	
18	Screening in newborn period is critical		X		X
19	Public health infrastructure existing	X		X	
20	Genetic counselling is possible	X		X	
21	Treatment risks explainable	X		X	
22	Limitations of the screening explainable		X		X

Table 1. Application of the 22-item extended framework of Wilson-Jungner criteria to advocate agammaglobulinemias (e.g. XLA) and inherited hemophagocytic syndromes (e.g. FHL3) to be considered for implementation in newborn screening programs (14).

AIMS

2.1 General research aim

The general aim of the work presented in this thesis was to develop and evaluate novel diagnostic assays to be used for newborn screening of severe primary immunodeficiencies, including SCID, XLA and FHL3 due to an *UNC13D* inversion.

2.2 Specific scientific aims

Publication 1: To design, develop and benchmark a triplex high-throughput real-time quantitative PCR assay for simultaneous detection of TREC, KREC and ACTB copy numbers in single neonatal dried blood spot punches.

Publication 2: To evaluate the feasibility and usefulness of an IgA-protein screening assay for identification of primary immunodeficiency diseases in newborns.

Publication 3: The development and evaluation of a combined newborn screening test for familial hemophagocytic lymphohistiocytosis due to an *UNC13D* inversion, as well as severe T and B cell immunodeficiencies.

Publication 4: To improve the performance of the TREC-KREC-ACTB screening assay from Publication 1, to study its performance in neonatal samples from Down syndrome patients, and to develop a second-tier test for Trisomy 21.

Publication 5: The assessment of the performance of the TREC-KREC-ACTB screening assay in predicting the clinical phenotype of infants with DiGeorge syndrome and to engineer a second-tier test for the 22q11 microdeletion.

2.3 Translational aim

The translational aim of this thesis was to promote the concept of newborn screening for several severe PID in one assay, and to motivate local prospective screening studies to be conducted.

HUMAN SUBJECTS, MATERIALS AND METHODS

3.1 Study populations and biobanked patient samples

The included PID patients and their stored neonatal Guthrie card samples have accumulated over many years. Most patients were treated or followed at the immunodeficiency or hematology hospital wards in Huddinge, Gothenburg or Lund (all Sweden), or in Leipzig and Freiburg (both Germany). All patients or their parents gave informed consent to obtain, use and store DBS and other available biological material. Ethical votes were obtained at Karolinska Institutet, Stockholm, Sweden, and the Medical Chamber of Saxony, Dresden, Germany. Guthrie card samples from the included patients, as well as storage-time matched anonymized control cards were obtained from storage at room temperature or at +4°C, and were subsequently biobanked in individually sealed bags containing a silica humidifier at -80°C for long-term storage.

For prospective screening studies, a fraction of the regular neonatal screening card (Whatman 903 or PerkinElmer 206 paper) was allocated for the TREC-KREC-ACTB or TREC-KREC-UNC13Dwt assay at the Centrum för Medfödda Metabola Sjukdomar (CMMS) in Stockholm. In Leipzig, a separate screening card (Whatman 903 paper) was provided to the participating maternity clinics. Further information about the included study populations and dried blood spot specimen is included in the methods sections of publications I-V.

3.2 Extraction of DNA from dried blood spots

During initial investigations and for verification of disease-causing mutations in DBS, several 3.2mm disk punches were processed using the Qiagen QIAamp DNA Mini Kit according to the manufacturer's instructions. For newborn screening purposes, a protocol published by Mei Baker was applied (23), as indicated in publications I, III, IV and V. This protocol consists of the assay reagents included in the Qiagen Generation Capture Kits, and releases DNA from the DBS by heat-incubation at 99°C in a 96-well PCR plate. For the newly developed SCREEN-ID kit-like assay, a proprietary rinse solution was developed at TRM Leipzig, which is similar in terms of chemical composition with the above mentioned commercially available reagents.

3.3 Elution of proteins from dried blood spots

Protein-containing eluates from DBS were prepared as described in publication II. In detail, single 3.2mm disk punches from stored Guthrie cards were distributed into single wells of deepwell-plates and incubated in 450 µl of phosphate-buffered saline (PBS) with 0.5% Tween20 under varying conditions for temperature (4°C – 37°C) and elution time (1 h – 7 days). To determine the most efficient elution procedure for IgA from DBS, serum IgA levels of freshly collected blood specimens were compared with the IgA levels of eluates from dried blood filter cards, prepared using the same blood samples. The serum levels of IgA were determined by routine nephelometry, while the IgA levels in the DBS eluates were determined by ELISA.

3.4 Preparation of plasmid standards for PCR-based quantitation

A 'TREC-KREC-TRAC' plasmid (pCR2.1-TOPO, TREC signal joint insert: 380 bp, TRAC insert: 367 bp, KREC signal joint insert: 168 bp) was kindly provided by Alessandra Sottini and Luisa Imberti, and a 'beta-actin' (ACTB) plasmid (pCR2.1-TOPO, human beta-actin DNA insert: 348 bp) was obtained from the lab of William J. Grossmann and Donna K. Mahnke. The UNC13Dwt plasmid was generated from amplified human whole blood DNA, sub-cloned into the pCR 2.1-TOPO vector (Life Technologies), using primers detailed in publication III.

Following transformation and cloning of each plasmid in competent E. coli cells, the bacteria were harvested from culture and plasmid DNA was isolated using the Qiagen Plasmid Midi Purification kit according to the manufacturer`s instructions. Subsequently, the plasmid DNA was digested using Xho I restriction enzyme, and extracted on a 1% agarose gel (130V, 40 minutes) before purified using a Qiagen Gel Extraction Kit. Plasmid stock solutions were prepared according to the calculated molecular weight of each plasmid, and stored in H20 supplemented with yeast tRNA (Ambion) at -25°C.

3.5 Real-time multiplex quantitative PCR reactions

During normal development of B and T cells, their immune receptors will undergo rearrangement processes to shape their specificity, resulting in genomic excision products. The rearrangement of the T-cell receptor (TCR) gene segments, that enables the diversity of the TCR, gives rise to T-cell receptor excision circles (TRECs) and the rearrangement of the kappa light-chain locus that contributes to antibody diversity of B cells will lead to the formation of

kappa-deleting recombination excision circles (KRECs). For amplification of the signal-joints of TRECs and KRECs, primers and hydrolysis (TaqMan) probes published by Alessandra Sottini were used (30), and for amplification of ACTB, primers and hydrolysis (TaqMan) probes published by Daniel Douek were used (31). The primer and hydrolysis (TaqMan) probes used in publications III for UNC13Dwt, in publication IV for the *PTBP1* gene and the *KCNE1* gene, and in publication V for *TBX1* gene quantitation were designed in-house as detailed in the respective publications.

As for publication I, we have been using a commercially available qPCR master mix from LifeTechnologies and the real-time qPCR was performed on ABI 7500 or ViiA7 instruments. With regard to publications III, IV and V, we have been placing a research and development service order at Affymetrix Incorporated for the allocation of a custom master mix for multiplex qPCR with inhibitor-rich DNA templates as typically obtained from DBS. This master mix contains a random-mutation-derived hot-start Taq DNA polymerase with similar properties as the Hemo KlenTaq polymerase, as well as nucleotides with an optimized dUTP:dTTP ratio, Uracil-DNA Glycosylase and ROX passive reference dye in a proprietary reaction buffer.

3.6 Newborn screening assays and kit versions

The TREC-KREC-ACTB newborn screening assay described in publication I is referred to be ‘version 1.0’, prior to a series of modifications aiming to (i) improve the assay resolution in the low-copy range of TREC and KREC absolute quantitation in DBS, as well as (ii) to ease the overall handling of the screening assay and to reduce the hands-on-time. As depicted in section 3.5, a

custom master mix containing an optimized Taq DNA polymerase, as well as improved concentrations of TREC, KREC and ACTB primers and hydrolysis (TaqMan) probes determined by primer-limiting assays, has been developed. These new core reagents allowed reducing the fraction of technically-determined cutoff values for TREC and KREC copies due to better low-copy range resolution in between patient samples and healthy newborns (Figure 1).

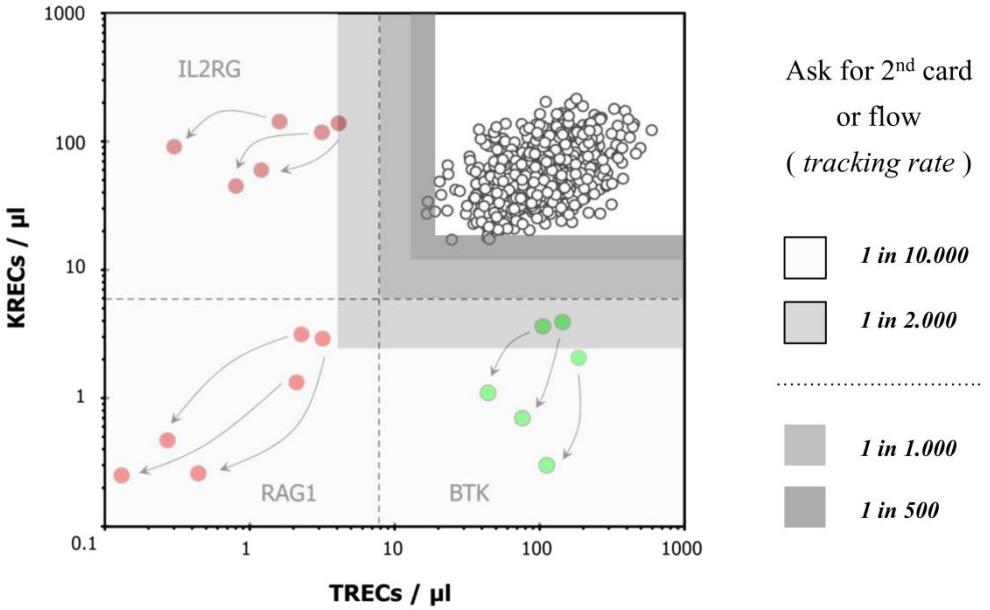

Figure 1. Improvement of the low-copy range resolution of the TREC-KREC-ACTB assay in comparison of copy numbers obtained from SCID patients (*IL2RG* gene or *RAG1* gene mutations) and XLA patients (*BTK* gene mutations) either in the 'version 1.0' assay used in publication I (higher TREC and KREC values, respectively) or the 'version 2.0' assay used in publications III, IV and V (lower TREC and KREC values, respectively). Patient samples in filled circles, healthy newborns in open circles.

During the process of evaluating the TREC-KREC-ACTB screening assay in local prospective screening studies, it became apparent that the liquid-handling, the overall processing time and the related working steps require optimization to further improve the applicability for mass screening of this assay. Thus, we have been designing a hitherto unpublished kit-like 'version 3.0' assay that reduces the required liquid-handling to a single working step in replacement for short centrifugation steps, and have combined all required reagents and plastic ware in a storable format (Figure 2). The assay is available on the basis of a material-transfer-agreement from the TRM Leipzig.

3.7 Mutation-verification by Sanger sequencing

All PID patients included in papers I, II and III were confirmed by Sanger sequencing for the presence of known disease-causing mutations. Eluted genomic DNA from the dried blood spot punches was used to amplify the coding regions of the intended PID genes, with primers obtained from the RAPID database (32). Subsequent to gel electrophoresis, PCR products were purified using MSB Spin PCRapace columns from Stratec, and were sent for direct sequencing to the IZKF core unit at the University of Leipzig.

3.8 Digital PCR product analysis

Following PCR with sequence specific primers and gel electrophoresis, PCR products were purified as indicated above. Subsequently, to investigate integrity and size of the PCR amplicons, separated PCR products were analyzed using DNA microfluidic-chips on a 2100 Bioanalyzer (both Agilent Technologies).

Figure 2. *SCREEN-ID* kit-like assay version for quantitative analysis of (i) TREC-KREC-ACTB or (ii) TREC-KREC-UNC13Dwt copies in neonatal DBS. The kit consists of pre-arranged 96-well plates containing a DNA-elution solution in the 'Elution plate', and qPCR reagents and plasmid standards for real-time qPCR in the 'qPCR plate'; both plates can be stored frozen at -25°C for up to 6 months. Only a single pipetting step is required to wet DBS with a rinse solution in the 'Filter unit'. All further assay steps rely on centrifugation of the plates containing DBS and assay reagents. Control card specimen are allocated to mimic T-cell deficient (TCD), B-cell deficient (BCD) or T- and B-cell deficient (TBCD) patient samples.

3.9 Statistical analyses

Comparison in between groups and correlation analysis was performed using XLSTAT 2013 (Addinsoft). Fisher´s exact test was used to identify differences between groups concerning categorical data and the Mann-Whitney U tests were used for continuous data. Statistical significance was defined as a p-value < 0.05. ROC (receiver operating characteristic) curve analyses were performed using standard settings in MedCalc version 14 (MedCalc Software).

RESULTS AND DISCUSSION

4.1 Rationale for newborn screening of severe PID

The earliest possible diagnosis of severe PID improves their prognosis and treatment efficiency significantly (33-35). This stems primarily from the possibility to initiate early preventive measures to avoid infection, and to prevent iatrogenic damage, e.g. caused by the administration of recommended vaccinations, such as the rotavirus vaccine (live vaccine), which is safe for immunocompetent infants (36,37). The prevention of such complications significantly improves overall survival before and after performing curative hematopoietic stem cell transplantation (HSCT), gene therapy (GT) or supportive enzyme replacement therapy or immunoglobulin replacement therapy (33). Given the special importance of an early pre-symptomatic diagnosis of patients with severe PID, the implementation of newborn screenings seems useful (14).

4.2 DNA-based newborn screening assays for severe PID

When applying established criteria in decision-making processes in newborn screening, one has to distinguish in between the target disease to be excluded and the applicable screening-biomarker itself. While in a number of metabolic diseases the respective screening marker is directly attributable to the pathogenesis of a target disease (e.g. in biotinidase deficiency or classic galactosemia), the requirements for screening markers for severe PID are more demanding. As SCID and XLA are pathogenetically and immunophenotypically

characterized by the absence of T- and/or B-lymphocytes, the target disease for newborn screening of these severe PID should be defined as ‘pronounced neonatal deficiency of autologous T- and/or B-lymphocytes’. In the evaluation of different laboratory methods for the detection of these PID, the measurement of episomal excision products of lymphocyte receptors (TRECs and KRECs) has prevailed, as this method, both in terms of test quality and practical feasibility, is consistent with prospective screenings (38). When applying the evaluation criteria of population-based screening tests to the measurement of TRECs and KRECs for the detection of the pronounced neonatal deficiency of autologous T- and/or B-lymphocytes, the implementation as newborn screening has been viewed positively (14). In several U.S. states, population-based screening programs for pronounced neonatal deficiency of autologous T-lymphocytes in newborns were initiated successfully in 2008, and now some countries in the European Union have begun their own pilot projects (Germany, Sweden, France, UK and Italy) (39,40).

The principle of measuring episomal excision products of lymphocyte receptors is based on the naturally occurring recombination and affinity maturation of the T-cell and B-cell receptors (41). Parts are excised from the germline DNA of the immunoglobulin genes that are not involved in the recombination process of the antigen receptors. While T-lymphocytes in the thymus initially excise portions of the δ-locus in order to then recombine the α-locus, so-called TRECs are created, which spontaneously form circular DNA fragments that are not replicated further during cell division. Following a similar principle, a deletion occurs in B-lymphocytes in the bone marrow during the Vk-Jk rearrangement, in the process of which KRECs are formed. Since these molecular processes are inter-individually uniform, a uniform PCR strategy can be applied to determine the presence and number of copies of TRECs and KRECs in dried blood samples

from neonates (publication I). During the formation of circular DNA fragments, a unique sequence region (so-called signal joint) is created, which means a quantitative PCR method can be used to detect only the TREC and KREC fragments already excised from the germline DNA, so that a direct conclusion about the maturation of T- and B-lymphocytes that has taken place is possible. Given that with this method no individual sequence information is collected, the PCR method is most appropriately comparable to virological pathogen-load diagnostics. Like the established analytics of regular newborn screening, such a PCR method, however, becomes subject to the scope of genetic diagnostics acts. In other words, patients must be informed in writing by medical staff with proven qualifications in the field of genetic counseling.

For the measurement of TRECs and KRECs in newborns, the use of 3.2 mm or 1.5 mm punches from regular dried blood spot cards (e.g. Whatman 903 paper) has proven most useful. These can be obtained in the time frame of the established logistics of newborn screening (Figure 3). At the screening lab, TRECs and KRECs are then detected in the sample material. Either relative endpoint-PCR or absolute quantitative real-time PCR methods can be used. The use of detection probes allows for an increase in the specificity of the amplicons to be detected and the simultaneous measurement of several different amplicons (multiplexing), which further improves cost-efficacy (Figure 3). In the analytical chemistry to be used, one can choose from hydrolysis probes in the 5' nuclease assay (e.g. TaqMan; from LifeTechnologies), time-resolved fluorescence resonance energy transfer (TR-FRET, e.g. from PerkinElmer) and iso-dG/iso-dC-labeled oligonucleotides with fluorescent dyes and quenchers (e.g. Plexor; from Promega). As experience from publications I, III, IV and V has shown, a two-step protocol is already sufficient for purifying inhibitor-free and concentrated DNA templates when compared to a direct elution from dried

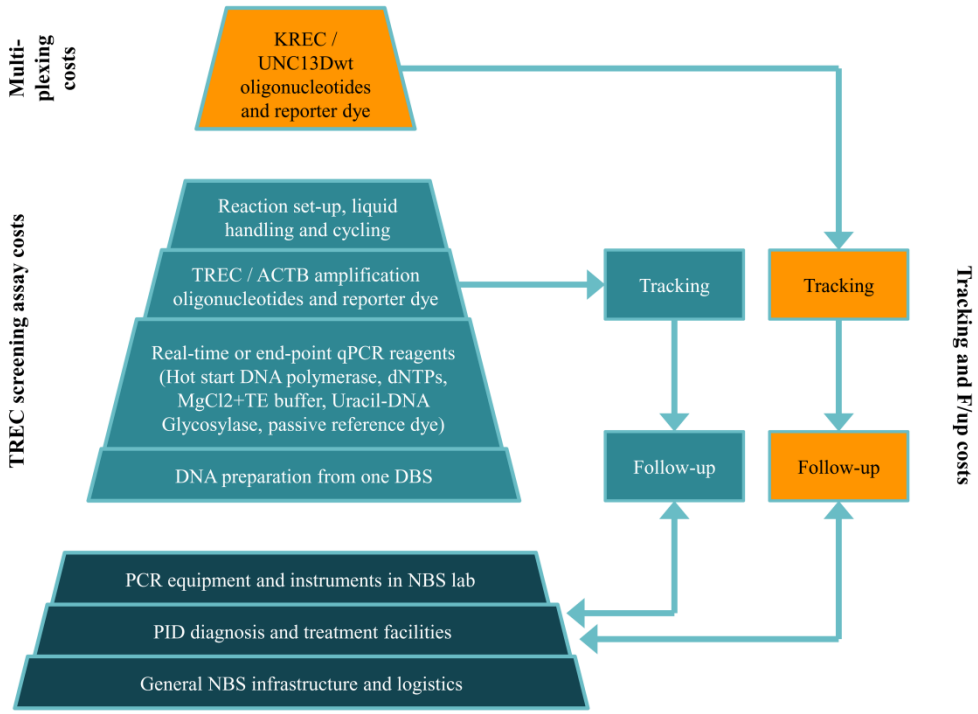

Figure 3. Schematic composition of partial screening costs in a TREC-only and multiplexed TREC+KREC newborn screening assay.

whole blood. Thus, particularly in the low-copy range, a better resolution can be achieved for the screening test (Figure 1). This is particularly relevant with regard to the determination of an appropriate diagnostic threshold (cut-off), as congenital T- or B-lymphopenias are not observed solely in patients with SCID or XLA (42). A TREC–KREC screening test that offers a good resolution for low copy numbers, allows for the implementation of a diagnostic threshold defined by the user in the screening lab, thus making it possible to differentiate "typical" SCID and XLA patients from those with borderline screening results observed in profound combined immunodeficiency diseases (Figure 4).

Figure 4. Relevance of the chosen cutoff values for TREC and KREC copy numbers on a population-scale screening basis with regard to expected SCID and PCID entities.

For a routine TREC–KREC screening, it is necessary to evaluate the test characteristics firstly in the local newborn population within a routine screening lab, and then to keep on validating them continuously. Usually, the copy numbers of TRECs and KRECs in the overall population are not normally distributed, so that 5,000 to 10,000 newborn samples should be evaluated in order to determine suitable diagnostic cut-offs in the low copy number range and to be able to estimate the repeat rate of the screening in this range. We have been performing such analyses using a TREC-KREC-ACTB screening assay as described in publications III, IV and V (Figures 5-1 and 5-2).

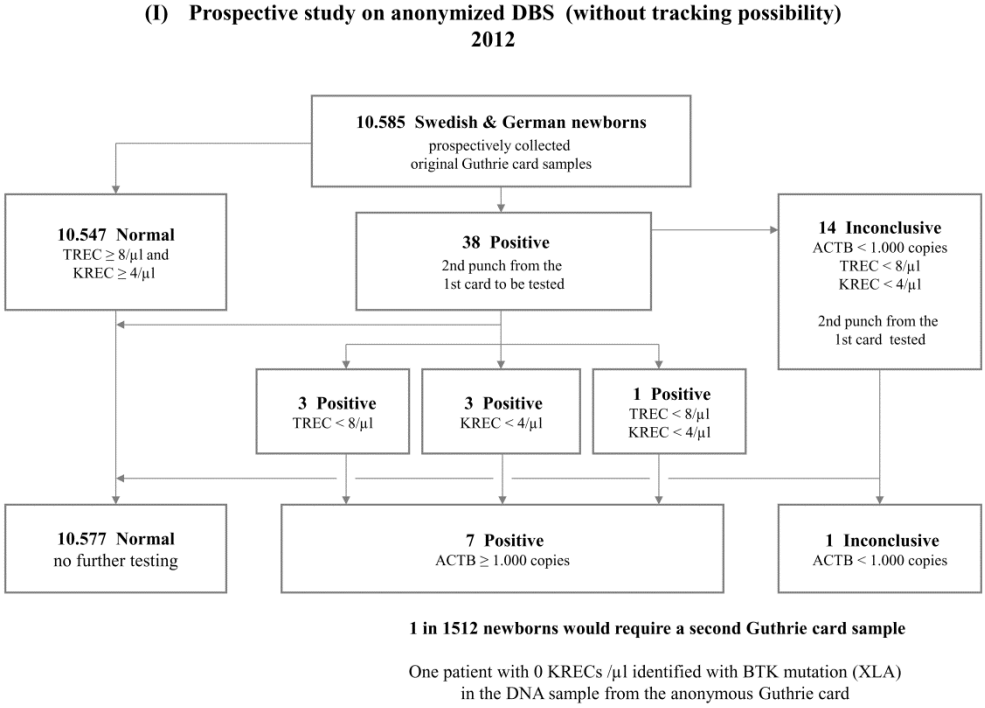

Figure 5-1. Establishing population-acceptable diagnostic cutoff values for TREC and KREC copy numbers using the assay described in publications III, IV and V.

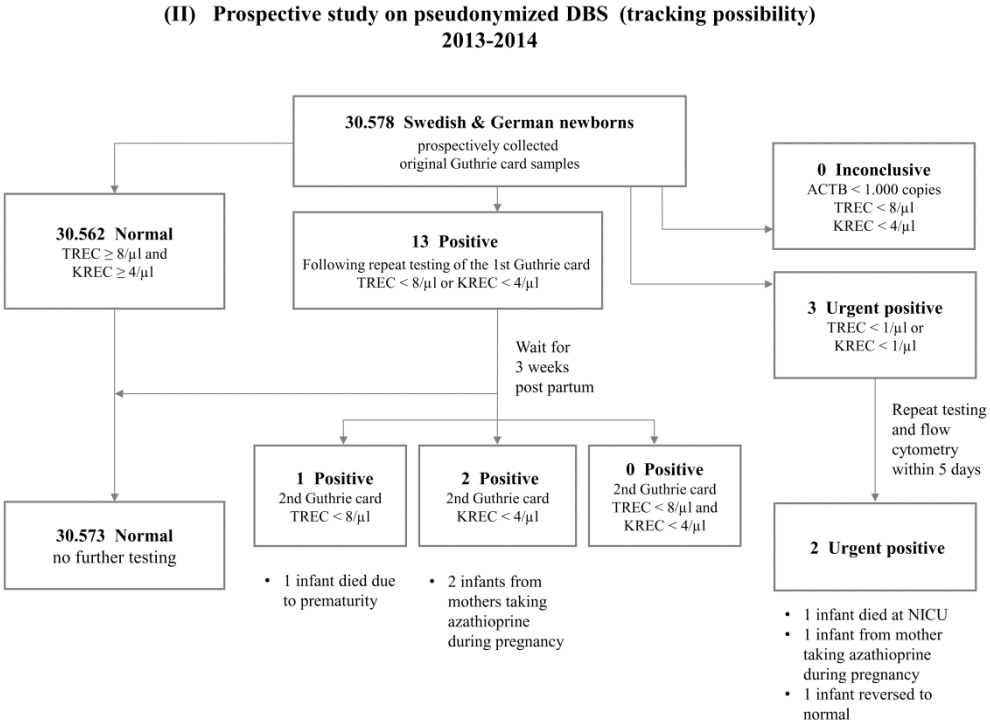

Figure 5-2. Outcome of two harmonized, prospective TREC-KREC-ACTB newborn screening studies in Stockholm and Leipzig; updated August 2014.

Currently, most screening tests used in the U.S. and Europe have been developed in-house, which makes it difficult to harmonize the methodology and transfer diagnostic cut-offs (Figure 5-2). The dried blood cards provided by the Center for Disease Control and Prevention (CDC) for the purpose of collaborative trials have been validated only in terms of the T-cell numbers, which means they will yield incomplete results when used for TREC–KREC screening tests. Therefore, for the continuous validation of the TREC–KREC screening test, artificial control samples should be produced (1: T- B+, 2: T+ B-, 3: T- B-, 4: T+ B+); this can be achieved by the depletion of T- and/or B-lymphocytes, e.g. in

umbilical cord blood as donor material. Alternatively, EBV cell lines can also be used in this context that carry genomically stable copies of the TREC or KREC signal joint. When selecting an absolute quantification mode, it is preferable to document the measured copy numbers of these control samples prospectively to complete the required quality assurance at the screening laboratory.

Depending on established diagnostic thresholds, prospective TREC–KREC screening results can be considered "normal" (equal to or above the thresholds for TRECs and KRECs) or "positive" (below the cut-offs for TRECs and/or KRECs) (Figures 5-1 and 5-2). In the event of a positive test result of the first dried blood spot punch, the lab runs an internal repeat test with two other punches from the same Guthrie card. The tracking process is initiated only if the two punches also yield positive test results. The proportion of newborns in the overall population for whom a separate, second Guthrie card has to be submitted is expressed as the tracking or re-call rate. The difference between repeat and tracking percentages is primarily due to pre-analytical factors, such as the method of producing the dried blood card and the selection of the punch position on the card. The selection of appropriate diagnostic thresholds should, however, also be based on these percentages to ensure that the screening test remains reasonable with respect to the overall population.

4.3 Tracking process for severe PID

Tracking of newborns is usually initiated in case of repeatedly positive test results for TREC and/or KREC copy numbers after examination of at least three independent dried blood punches from the first Guthrie card submitted to the screening lab (Figures 5-1 and 5-2). The coordination and documentation of the

tracking in this case lies in direct responsibility of the screening laboratory that has performed the initial TREC–KREC screening test, but can also be passed to a specialized tracking center.

Firstly, the obstetric unit (‘doctor–doctor’ interaction) and the parents of the newborn (‘doctor–patient’ interaction) should be contacted to obtain additional information on the status of the child. Since repeatedly positive TREC and/or KREC test results can occur especially in preterm infants (<32nd week of pregnancy), children with syndromic diseases (e.g., trisomy 21 or 22q11 microdeletion), metabolic diseases (such as propionyl-CoA carboxylase deficiency or methylmalonic aciduria), and severe congenital infections or malformations with subsequent lymphocyte extravasation (e.g., neonatal sepsis or gastroschisis), it is important to gather and compile additional examination findings to evaluate a suspected diagnosis of a severe congenital immunodeficiency (publications IV and V, 42). If no TREC and/or KREC copies can be detected reliably at all in the three repeat samples of the first Guthrie card, one has to assume an "urgently positive" finding. In these rare cases, one should immediately counsel clinical consultation on the strong suspicion of SCID and/or XLA. Within the prospective study on anonymized DBS in 2012 (Figure 5-1), we have been identifying a neonate with <1/µl copies of KRECs, which later on proved to harbor a *BTK* gene mutation resulting in XLA (Figure 6) (43).

In any other case, a separate second Guthrie card is immediately ordered to do a follow-up on the newborn. In the case of preterm infants, the second dried blood spot should probably not be sent in before the calculated date of delivery. If the second Guthrie card confirms the initial positive screening result, a persistent T- and/or B-lymphopenia should be assumed and an experienced pediatrician,

preferably associated with an immunodeficiency center, should be consulted. To this end, the screening laboratory will compile all additional available test results, especially those of the regular newborn screening that might suggest the existence of a metabolic disorder.

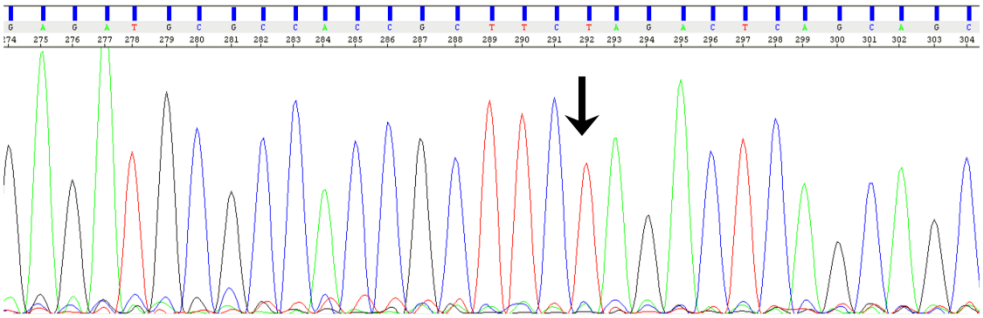

Figure 6. Electropherogram of the disease causing XLA point mutation in Exon 15 of the *BTK* gene (c.1480C>T, p.Q494X), leading to a premature stop codon in the TK domain. This mutation has been described before in XLA patients with an ethnic origin located in Serbia (43).

4.4 Immunophenotype in premature newborns

Infants born before 37 weeks of gestation may suffer from a variety of cardiovascular, neurological, metabolic and gastrointestinal complications, in addition to an increased susceptibility to respiratory and urinary tract infections (42). Although many risk factors have been linked to preterm birth, the multifactorial causes remain unresolved in most of the affected infants, limiting the availability of effective interventions. In developed countries, the birth rate of premature newborns is about 5 to 12 % with a tendency to rise, posing a

concern for newborn screening programs in particular and for public healthcare systems in general. Thus, if dried blood spot samples are taken before the 32nd week of gestation, a second analysis later on for newborn screening tests of metabolic disorders to distinguish abnormal results of genetic origin from physiological immaturity often is required (42).

A higher rate of abnormal test results for preterm infants has previously been observed in the TREC assay (44). In this context, the immaturity of the immune system as a temporal effect of the gestational age may well be distinguished from specific factors related to the medical condition of preterm newborns. These factors are likely to include congenital anomalies, infectious complications and disturbed metabolic and endocrinologic conditions that may contribute to thymic stress and reduced output of recent thymic emigrants (45). Few data on reference ranges for TREC and KREC copy numbers has been published for premature infants, resulting in different practices for repeat testing of samples and referral of patients. In groups of premature newborns sorted by gestational age in weeks, analysis of TREC and KREC copy numbers tended to be consistent until 28 weeks age of gestation, though outliers with the detected frequencies would have an impact in regular newborn screening programs (Figure 7). Thus, samples from premature infants are basically eligible to be analyzed by excision circle assays, and might also not have to be retested at a later stage if cut-off values to have been exceeded initially.

4.5 Follow-up diagnostics

The follow-up diagnostics of newborns suspected of a severe PID have to meet several requirements at the same time. On the one hand, the repeatedly

positive screening result has to be confirmed by way of exploratory (complete blood count) and detailed cytological tests (flow cytometry). On the other hand,

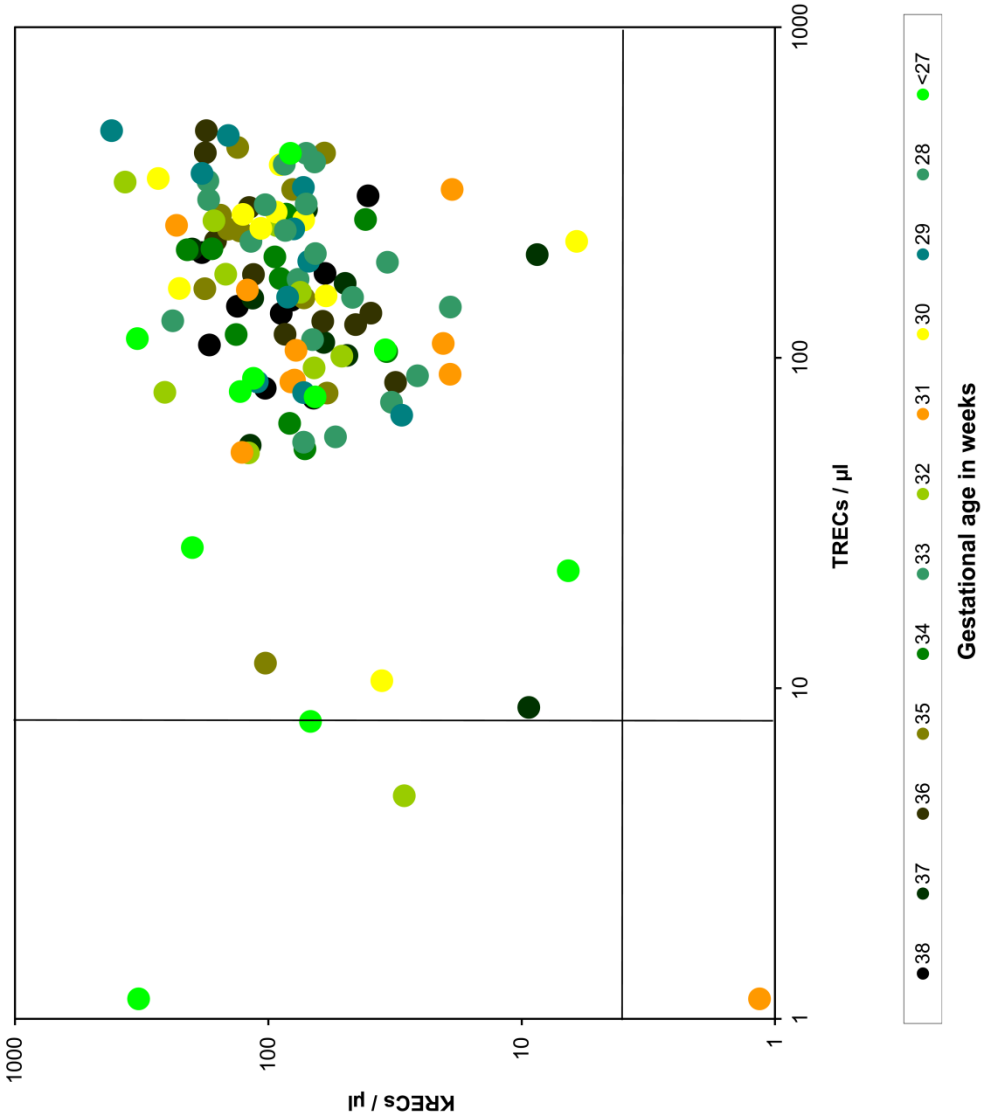

Figure 7. TREC and KREC copy numbers in neonatal DBS of premature born infants. Cutoff values: TREC <8 copies, KREC <4 copies.

conclusions should be drawn from immunofunctional and molecular genetic tests about the pathogenetic background of each patient in preparation of an individualized treatment plan. The need for such patient-centered follow-up diagnostics is derived from the considerable molecular heterogeneity of severe PID with numerous "private" mutations.

Flow cytometric lymphocyte typing is to provide a confirmation of the screening suspicion by means of a methodologically independent, peripheral whole blood examination and at the same time describes the composition of lymphocyte subpopulations, including recent thymic emigrants (RTE), and excludes a maternal engraftment with CD45RO+ T-cells. While in patients with "typical" SCID phenotypes or agammaglobulinemia, the almost complete absence of autologous T- and/or B-lymphocytes (< 300 T-cells and/or < 100 B-cells per microliter of whole blood) already is a clear diagnostic signal, reduced T-cell numbers (300–1500 T-cells per microliter of whole blood) may be detectable in clinically relevant PCID phenotypes (Figure 4). However, normal T-cell numbers can also be observed simultaneously with reduced B-cell numbers in newborns with "delayed-onset" ADA SCID, indicating that a SCID phenotype has always to be considered in connection with an isolated decrease in KREC copy numbers (46).

Immunophenotyping often fails to provide a definitive assessment of the functional relevance of an anticipated severe PID, especially when the flow cytometric analysis or clinical examination suggests the existence of an "atypical" phenotype. Therefore, for the completion of flow cytometric tests, (i) the lymphocytic proliferation and stimulation ability, as well as (ii) the functionality of the innate diversity of the immune repertoire should also be examined in-vitro.

4.6 Confirmatory genetic testing

Currently, more than 18 monogenetic defects have been described, resulting in a phenotype of severe combined immunodeficiencies [47]. Likewise, primary agammaglobulinemia with some missing B-lymphocytes can be traced back to more than six genetic defects (1). In terms of pathogenesis, the underlying defects can be associated with maturation disorders, especially of the lymphatic lineage in the bone marrow, as well as in primary and secondary lymphatic tissues. Flow cytometric evaluation of the peripheral blood makes it possible for SCID patients to be classified according to the T- B+/- NK+/- system, which can further indicate the underlying genetic defect (1). The molecular and cellular functional heterogeneity of severe combined immunodeficiencies is also evident in the clinical treatment experience, which led to the application of "gene-specific" protocols in conditioning and stem cell transplantation or gene therapy for SCID patients (48,49).

While the decision for stem cell transplantation has so far been based on the clinical course, as well as cellular and immunofunctional tests, the pre-symptomatic early identification of newborns with severe PID poses a new challenge for the algorithms of treatment decision. Immunological function testing of infants, in particular, is struggled by the lack of sufficient reference ranges, because age-weighted control samples are not regularly available. Therefore, molecular genetic tests are of special importance for newborns with neonatal deficiency of autologous T-lymphocytes and a suspected SCID or PCID phenotype. At the same time, the initiation of a curative therapy should not be delayed, neither by the time required for molecular testing, nor by the absence of a final genetic diagnosis. In newborns with severe neonatal deficiency of autologous B-lymphocytes and suspicion of agammaglobulinemia, the

transferred maternal immunity, which usually lasts up to 6 months following birth, can help to sustain the time until a complete genetic diagnosis prior to the start of treatment is made (50).

Currently, molecular genetic testing in a clinical context is predominantly performed through selection of annotated candidate genes for the suspected congenital immunodeficiency. However, the limitation to candidate genes represents a problem with regard to the molecular heterogeneity and the known overlapping effects in the clinical phenotype, especially in the case of a sequential approach ("gene-by-gene"). More recent molecular genetic methods can partially or completely avoid these disadvantages, but sequence-analytical challenges in view of ever increasing amounts of data currently represent the primary obstacle to a timely reporting of results. On the one hand, specific exon enrichment (e.g. HaloPlex system) makes it possible to accommodate individualized diagnostic panels for up to 250 candidate genes on a single microfluidic gene chip. On the other hand, the third generation of sequencers holds out the promise of a cost- and time-efficient analysis of entire transcriptomes, exomes or even genomes (51). The advantage of transcriptome- or exome-wide tests is the ability to simultaneously examine comprehensively known candidate genes sequentially and to identify new disease-associated genes for PID. In view of the evidence-based justification for lifelong supportive or invasive therapies, such as a hematopoietic stem cell transplantation, this will become increasingly important (52).

CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this thesis was to present newly developed diagnostic assays to be used for newborn screening of severe primary immunodeficiencies, including SCID, XLA and FHL3 due to an UNC13D inversion. The conclusions drawn from the individual publications in the thesis are:

1. A triplex real-time quantitative PCR assay for measurement of TREC, KREC and ACTB copy numbers in dried blood spot specimen is a suitable tool for identification of PID characterized by a severe T and/or B cell lymphopenia at birth, without disclosing functional B cell immunodeficiencies with normal absolute B cell counts (Publication I).

2. Analysis of IgA-protein levels in Guthrie card samples is not effective as a screening tool for PID due to maternal transfer of IgA. Other protein based screening assays, however, might be beneficial in NBS for severe PID (Publication II).

3. Combination of the TREC-KREC screening assay with measurement of the UNC13Dwt allele in exchange for ACTB allows identifying patients with FHL3 due to an UNC13D inversion. This indicates that the TREC-assay can be multiplexed with two more relevant biomarkers for early identification of severe PID (Publication III).

4. An improved version of the TREC-KREC-ACTB screening assay, developed in Publication I, allows for reduction of TREC and KREC cutoff levels without reducing the sensitivity to identify newborns with SCID and XLA. Neonates with Down syndrome present only a small fraction of positive screening results and exclusion is possible based on a qPCR second-tier assay (Publication IV).

5. Retrospective analysis of Guthrie card samples from DiGeorge patients using the TREC-KREC screening assay identifies a high proportion of neonates with T cell lymphopenia and subsequently increased viral infections in early life. The development of a second-tier qPCR assay allows identifying newborns with 22q11 microdeletions in dried blood spots (Publication V).

The use of excision circle assays has not proven to be helpful for early detection of antibody deficiencies such as common variable immunodeficiency (CVID), Hyper-IgM syndromes (HIGM) or selective IgA deficiency (IgAD), nor would these assays be expected to identify patients with complement deficiencies. (42) Moreover, disorders that are detected only partially by TREC / KREC assays will require alternative screening approaches to be eligible for preventive programs.

To overcome the difficulties to find common delineators for PID screening, it might be useful in the future to develop assays that measure disease-specific biomarkers. A variety of potential biomarkers for PID have been proposed before (42), and suitable technological platforms and nanomaterials for

immunoassays and enzyme activity assays have been developed in recent years. Our group has previously demonstrated that analyzing a multitude of serum protein levels is also feasible using reverse phase protein microarrays, a technique that can be adapted for the screening of C3 complement deficiency in eluted dried blood spot cards and might also be expanded on low abundance complement factor deficiencies. Combining this protein microarray platform with digital microfluidics in the future has furthermore the potential to markedly reduce the amount of reagents and time consumed.

One of the technologies that could be employed in future newborn screening is based on proximity ligation of unique nucleic acid flags, which can be attached to diagnostic antibodies or designed to capture bacterial or viral recognition sequences directly (42). The proximity ligation assay (PLA) offers a possibility for multiplex analysis and quantitation of proteins in nanoliter volumes of crude sample mixtures with a sensitivity reaching the picomolar range.

Our future focus will be directed towards functional PID to be included in newborn screening assays, particularly defects of phagocytic function and PCID currently not detected in excision circle assays. The economical evaluation of the cost-effectiveness of novel technologies for neonatal screening is a challenging mission, as various factors – that may also underlie regional or country-specific characteristics – determine the outcome of such analyses (42).

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