

Clinical Communications

Time-dependent decline of T-cell receptor excision circle levels in ZAP-70 deficiency

Sneha Suresh, MD^{a,b}, Harjit Dadi, PhD^{a,c},
Brenda Reid, RN, MN^a, Linda Vong, PhD^{a,c},
Dennis E. Bulman, PhD^d, and
Chaim M. Roifman, MD, FRCP^{a,c}

Clinical Implications

- T-cell receptor excision circle (TREC) levels decline over time in patients with ZAP-70 deficiency. Current threshold levels for TREC analysis are unable to detect cases of ZAP-70 deficiency at birth.

TO THE EDITOR:

Measurement of T-cell receptor (TCR) excision circles (TRECs) as a screening tool has revolutionized the diagnosis and management of many T-cell primary immunodeficiencies by enabling early diagnosis.^{1,2} The screening test is based on quantitative analysis of a DNA byproduct of T-cell maturation, the δ Rec- Ψ J α TREC,³ which is produced late in T-cell maturation by the majority of $\alpha\beta$ T cells. TREC levels have been shown to correlate well with thymic output of new T-cell emigrants.^{1,3}

However, although screening has been a consistently reliable method to detect typical severe combined immunodeficiencies (SCID), it has been much less effective in uncovering a large number of T-cell immunodeficiencies, including combined immunodeficiencies (CID), hypomorphic SCID, as well as T-cell deficiencies affecting immune regulation.^{2,4} Of particular and unique interest are patients with ZAP-70 deficiency who typically present with clinical features indistinguishable from SCID. Immune assessment reveals normal lymphocyte counts albeit with paucity of circulating CD8+ T cells, whereas CD4+ T cells are dysfunctional and fail to expand in response to mitogens or antigens.⁵ This can be explained by the critical role of ZAP-70 in TCR-mediated signal transduction.⁶ On ligation of the TCR, ZAP-70 is activated after recruitment of phosphorylated immunoreceptor tyrosine-based activation motifs on CD3 ζ and through phosphorylation by Lck, an Src family kinase.⁷ Active ZAP-70 subsequently phosphorylates numerous downstream proteins, including LAT, SLP-76, and Cbl. This cascade mediates a rise in intracellular calcium that is required for enzymatic activity, regulation of cytoskeleton reorganization, as well as expression of multiple genes.

Unlike the typical dysplasia observed in SCID, the thymus in ZAP-70 deficiency appears morphologically intact with normal corticomedullary demarcation and the presence of fully developed Hassall's corpuscles.⁸ However, the corticomedullary ratio appears to be disturbed, and evaluation of the rate of TCR gene rearrangement at various stages of intrathymic maturation has revealed attenuated transition from double negative to double positive thymocytes.

Assessment of TREC levels in a few cases of ZAP-70 deficiency revealed diverse results ranging from low to normal.⁸⁻¹⁰ Newborn screening (NBS) in Canada over the past 5 years did not detect 6 cases of ZAP-70 deficiency (personal communication), but we have occasionally observed low TREC levels in older patients.⁸ This led to the speculation that TREC levels may decline over time in patients with ZAP-70 deficiency. This study explored this possibility by evaluating TREC levels at birth and subsequently during infancy.

Twelve patients with genetically confirmed ZAP-70 deficiency (NM_001079.3:c.1624-11G>A) were included in this study. Samples from 5 patients were sent to our laboratory for molecular diagnosis and were evaluated at only 1 time point as patients were not available for follow-up (designated as single assessments, Figure 1). Analysis of these samples was performed between 132 and 285 days of age (Figure 1). Only 1 of 5 patients had an abnormal TREC level, which was found in the oldest infant at age 285 days.

Seven patients were available for evaluation and long-term follow-up, including multiple assessments of TREC levels. Five of 7 patients had stored Guthrie cards that were used for retrospective analysis of TRECs (see this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org for detailed methods). These NBS-based TREC levels were above threshold levels, ranging between 173 and 2123 copies/3 μ L of blood (cutoff in Ontario is 75 copies/3 μ L blood). It is possible that the NBS TREC level of patient 1 could have been flagged during screening in a few, but not in most, other jurisdictions (states, countries) that use higher cutoff levels.⁹ All 7 patients had their first whole blood TREC analyzed between the ages of 10 days (patient 2) and 283 days (patient 4). In 5 of 7 patients, TREC levels ranged from 750 to 3070, all above cutoff levels (700 TRECs per 0.5 μ g DNA are considered normal levels). In the remaining 2 patients (patients 1 and 3), initial assessment revealed that whole blood TREC levels were abnormal at 120 and 225 days of age. Five patients had multiple whole blood TRECs analyzed, and in 4 patients, the levels declined over time but remained above normal threshold. Only 2 of 5 patients reached abnormal levels at 275 (patient 7) and 422 (patient 6) days of age, before hematopoietic stem cell transplantation (HSCT). One patient (patient 2) had a surprising rise in TREC levels at 79 days of age before transplantation.

Patients presented during infancy with features typical of SCID encompassing viral, fungal and bacterial infections, chronic diarrhea, and failure to thrive (Table E1, available in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org). Evaluation of the immune system was consistent with ZAP-70 deficiency in all patients assessed. Although CD3+CD4+ circulating T cells and CD19+ B cells were normal, CD3+CD8+ cells were markedly reduced and *in vitro* proliferative responses to mitogens were consistently flat. Immunoglobulin levels were within normal range in all patients (Table 1).

All but patient 2, who was diagnosed immediately after birth because of a positive family history, suffered serious pulmonary infections. *Pneumocystis jirovecii* pneumonia was isolated in 4 patients, but in 3 of 4 patients, other infectious agents were identified concomitantly, including parainfluenza (patients 1, 3, and 6), *Klebsiella pneumoniae*, and *Candida albicans* (patient 1) or *Enterobacter* *sp* (patient 3).

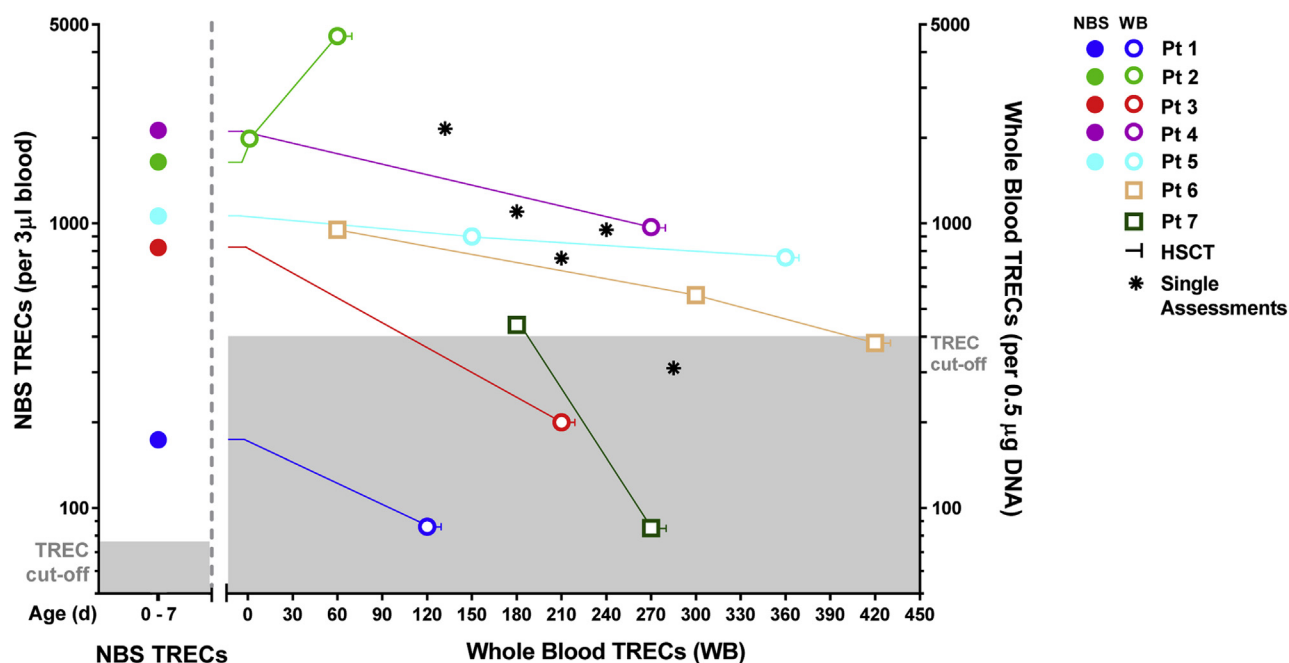


FIGURE 1. Measurement of T-cell receptor excision circle (TREC) levels in ZAP-70-deficient infants. TREC levels, a marker of T-cell maturation, were measured from Guthrie blood spot cards obtained shortly after birth by newborn screening (NBS, *closed circles*) and at various time points thereafter by whole blood TREC analysis (WB, *open circles and squares*). All initial assessments of TRECs were above the threshold/cutoff levels used to screen for severe combined immunodeficiencies (SCID) (*gray regions*). Seven patients received a hematopoietic stem cell transplantation (HSCT, $^{-}$). Measurements of TREC levels from ZAP-70-deficient infants who were unavailable for follow-up, and therefore evaluated at only 1 time point, are labeled as single assessments (*). Molecular genetic analysis confirmed a homozygous mutation in the *ZAP70* gene, NM_001079.3:c.1624-11G>A (g.98354447G>A), in patients 1-7.

TABLE I. Baseline immune laboratory investigations of patients with ZAP-70 deficiency at presentation

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age at diagnosis	7 mo	Birth	5 mo	9 mo	6 mo	5 mo	7 mo
WBC (N)	8.1 (6.4-13)	27 (7.2-18)	7.8 (6.7-14)	9.9 (6.4-13)	12 (6.4-13)	9.3 (6.7-14)	8.4 (6.4-13)
Lymphocytes (N)	2.1 (3.4-9)	2.4 (3.4-7.6)	4.6 (3.4-9)	4.0 (3.4-9)	3.9 (3.4-9)	4.8 (3.4-9)	3.4 (3.4-9)
Immunophenotyping (cells/ μ L)							
CD19 (N)	774	711	782	1230	814	631	904
CD3 (N)	1071	1050	2987	3930	2773	3579	2250
CD4 (N)	1044	984	2945	3900	2750	3554	2010
CD8 (N)	27	68	46	32	12	22	37
NK (N)	183	304	607	738	350	680	275
Immunoglobulins (g/L)							
IgG (N)	1.8	4.7 (2.8-14.8)	5.3	3.6	5.9	6.1	5.3
IgA (N)	0.4	0.1 (0.10-1.29)	0.5	0.9	0.7	1.2	0.9
IgM (N)	0.4	0.4 (0.12-1.25)	1.1	0.5	0.7	0.6	0.5
Responses to mitogens							
PHA (% control)	0.2	ND	0.2	0.17	1	0.5	0.1

N, Normal range; ND, not defined; PHA, phytohemagglutinin.

The variability in TREC levels among patients was not related to the *ZAP70* genotype as they all carried the same c.1624-11G>A mutation. To date, all patients received an HSCT and are alive and well.

We have demonstrated here that current threshold levels for TREC analysis are unable to detect cases of ZAP-70 deficiency at birth. However, we show a time-dependent and gradual

decline in TREC levels that may vary from several weeks to more than a year postnatally. This is consistent with our previous observation that identified an attenuation of gene rearrangements in thymocytes of ZAP-70-deficient thymi.⁸ Changing the threshold for whole blood TREC from 700 to 1000 would have captured most cases of ZAP-70 deficiency at a significantly earlier age. It is therefore plausible to use a

higher threshold in the proper clinical circumstance, or consider the use of different thresholds for ethnic groups at higher risk for certain genetic causes of profound CID or SCID.

^aDivision of Immunology and Allergy, Department of Pediatrics, The Hospital for Sick Children and the University of Toronto, Toronto, ON, Canada

^bDivision of IHOPE, Department of Pediatrics, University of Alberta, Edmonton, AB, Canada

^cThe Canadian Centre for Primary Immunodeficiency and The Jeffrey Modell Research Laboratory for the Diagnosis of Primary Immunodeficiency, The Hospital for Sick Children, Toronto, ON, Canada

^dCHEO Research Institute and Newborn Screening Ontario, Department of Pediatrics, University of Ottawa, Ottawa, ON, Canada

This work was supported by Immunodeficiency Canada's Distinguished Professorship in Immunology, the Program for Immunogenomics, and the Canadian Centre for Primary Immunodeficiency, the Jeffrey Modell Foundation, and Immunodeficiency Canada.

Conflicts of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication May 27, 2019; revised August 8, 2019; accepted for publication August 10, 2019.

Available online ■ ■

Corresponding author: Chaim M. Roifman, MD, FRCPC, Division of Immunology and Allergy, The Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada. E-mail: chaim.roifman@sickkids.ca.

2213-2198

© 2019 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaip.2019.08.018>

REFERENCES

1. Chase NM, Verbsky JW, Routes JM. Newborn screening for SCID: three years of experience. *Ann N Y Acad Sci* 2011;1238:99-105.
2. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. *JAMA* 2014;312:729-38.
3. Puck JM. Laboratory technology for population-based screening for severe combined immunodeficiency in neonates: the winner is T-cell receptor excision circles. *J Allergy Clin Immunol* 2012;129:607-16.
4. Grazioli S, Bennett M, Hildebrand KJ, Vallance H, Turvey SE, Junker AK. Limitation of TREC-based newborn screening for ZAP70 severe combined immunodeficiency. *Clin Immunol* 2014;153:209-10.
5. Arpaia E, Shahar M, Dadi H, Cohen A, Roifman CM. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking zap-70 kinase. *Cell* 1994;76:947-58.
6. Chan AC, van Oers NS, Tran A, Turka L, Law CL, Ryan JC, et al. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *J Immunol* 1994;152:4758-66.
7. Wange RL, Guitian R, Isakov N, Watts JD, Aebersold R, Samelson LE. Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. *J Biol Chem* 1995;270:18730-3.
8. Roifman CM, Dadi H, Somech R, Nahum A, Sharfe N. Characterization of zeta-associated protein, 70 kd (ZAP70)-deficient human lymphocytes. *J Allergy Clin Immunol* 2010;126:1226-1223.e1.
9. Roifman CM, Somech R, Kavadas F, Pires L, Nahum A, Dalal I, et al. Defining combined immunodeficiency. *J Allergy Clin Immunol* 2012;130:177-83.
10. Jilkina O, Thompson JR, Kwan L, Van Caesele P, Rockman-Greenberg C, Schroeder ML. Retrospective TREC testing of newborns with severe combined immunodeficiency and other primary immunodeficiency diseases. *Mol Genet Metab Rep* 2014;1:324-33.

ONLINE REPOSITORY**TREC DETERMINATION FROM WHOLE BLOOD**

T-cell receptor excision circle (TREC) levels from whole blood were quantified by polymerase chain reaction (PCR) as previously described.^{E1} Briefly, DNA was extracted from isolated peripheral blood mononuclear cells. Amplification reactions contained 0.5 µg genomic DNA, TaqMan universal PCR master mix (Applied Biosystems, Foster City, Calif), appropriate primers (900 nmol/L), and 6-carboxyfluorescein probes (250 nmol/L).^{E2} PCR (2 minutes at 50°C, followed by 95°C for 10 minutes, and then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) was carried out in an ABI PRISM 7900 Sequence Detector TaqMan system (Applied Biosystems). The number of TRECs was estimated by comparing the cycle threshold value against a standard curve (10² to 10⁶ copies of signal joint TRECs; provided by Dr. Daniel Douek, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda).

TREC DETERMINATION FROM GUTHRIE BLOOD SPOTS

Neonatal blood was collected onto Whatman 903 protein saver cards and sent to Newborn Screening Ontario, Ottawa, for analysis. A single 3.2-mm spot was punched from each sample, transferred to a 96-well U-bottom polypropylene microtiter plate, and washed twice with 20 mM Tris-HCl pH 9.0, 0.5% Triton-X, and once with 20 mM Tris-HCl pH 9.0 for 10 minutes at 37°C on a microplate shaker. DNA was eluted in 50 µL of Tris-HCl pH 9.0, 50 ng/µL yeast t-RNA at 98°C and subsequently stored at -20°C until completely frozen.

Dried blood spot (DBS) calibrators were created by serially diluting a minigene (IDT) constructed based on a TREC insert sequence^{E3} in sterile, charcoal-stripped, heat-inactivated fetal bovine serum (Sigma, St. Louis, Mo). Blood diluent was prepared from whole blood collected in sodium heparin collection

vials, spun at 3000 rpm in a centrifuge to remove the plasma layer, followed by 3 washes with sterile sodium chloride solution (Sigma) to remove residual anticoagulants. Endogenous TREC levels of the diluent were determined before using to ensure that TREC levels were undetectable. A small aliquot of the blood diluent was mixed with non-minigene containing fetal bovine serum to use as the severe combined immunodeficiency positive control. The diluted minigene was then mixed in a 1:1 ratio with the washed packed red blood cells and left to mix for 2-4 hours before spotting. A volume of 75 µL of mixed blood was applied to Whatman 903 protein saver cards and left to dry overnight at room temperature. An 8-point calibration curve run in triplicate along with patient DBSs.

Absolute quantification of TREC was determined by PCR. Forward and reverse primers were designed to flank the δRec-ψJa TREC-specific splice junction. Each 10 µL amplification reaction contained 5 µL DurAmp Master Mix (ThermoFisher, Waltham, Mass), TREC primer, and probe sequences in the following concentrations: 0.9 µmol/L each of forward TREC primer (TGCTGACACCTCTGGTTTTTGTAA) and reverse TREC primer (GCCAGCTGCAGGGTTTAGG), 0.25 µmol/L TREC-specific hydrolysis probe (Quasar670-ATGCATAGGCACCTGCAC-BHQ2Plus) (LGC Biosearch, Middleton, Wis), and 4.8 µL DNA eluate, and was performed with an Applied Biosystems ViiA 7 Real-Time PCR System using the following PCR cycling parameters: denature DNA for 1 cycle at 95°C (45 seconds) followed by 45 cycles of 95°C (30 seconds) and 60°C (1 minute 30 seconds). Absolute quantification was performed using the Applied Biosystems QuantStudio v1.2 Sequence Detection System software. Autobaseline and autothreshold setting were selected during analysis. For any single 384-well plate, we allowed the elimination of 8 of the 24 calibration points. The acceptable ranges set for the slope were -3.32 (±2 standard deviation [SD]), Y-intercept Cq value of 41 (±2SD), and R-2 value of 0.90 (±2SD). The TREC values were reported in units of TREC copies/3.2 mm DBS.

TABLE E1. Pretransplant clinical symptoms of patients with ZAP-70 deficiency in the first year of life

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age at ZAP-70 diagnosis	7 mo	Birth	5 mo	9 mo	6 mo	5 mo	7 mo
Clinical course							
<3 mo	Diaper dermatitis, chronic diarrhea	Healthy	GERD, intermittent diarrhea	Diaper dermatitis, oral thrush	Oral thrush, diarrhea	Repeated diarrhea	PJP pneumonia
3-6 mo	FTT, recurrent respiratory infections	Healthy	Respiratory failure (PJP, parainfluenza, enterobacter pneumonia)	Bilateral pneumonia	Oral thrush, chronic diarrhea, FTT	Chronic diarrhea, FTT	Chronic diarrhea
>6 mo	Rotavirus, diarrhea, PJP pneumonitis, parainfluenza, Klebsiella pneumonia, respiratory failure on HFOV	N/A	N/A	RSV pneumonitis, impetigo, eczema, oral ulcers	RSV pneumonitis, skin rash	PJP pneumonitis, parainfluenza pneumonitis, eczema	

FTT, Failure to thrive; GERD, gastroesophageal reflux disease; HFOV, high flow oscillatory ventilation; PJP, *Pneumocystis jiroveci* pneumonia; RSV, respiratory syncytial virus.

REFERENCES

- E1. Roifman CM, Dadi H, Somech R, Nahum A, Sharfe N. Characterization of zeta-associated protein, 70 kd (ZAP70)-deficient human lymphocytes. *J Allergy Clin Immunol* 2010;126:1226-12233.e1.
- E2. Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med* 2005;201:1715-23.
- E3. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 1998;396:690-5.