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Newborn screening for PIDs using both TREC and KREC identifies late occurrence of B cells

To the Editor,

Tuscany is the first region in Italy to have implemented a neonatal screening for congenital immunodeficiencies using both tandem mass spectrometry for early- and late-onset adenosine deaminase and purine-nucleoside phosphorylase deficiency¹ and multiplex real-time PCR for TREC and KREC quantification on dried blood spot (DBS).²

The screening program with TREC and KREC started on December 2013 and, based on the last data update of March 2017, it has screened a total of 18981 newborns in these first 3 years. We have had no cases of low or diminished TRECs but five cases of low/absent KRECs. The first newborn with absent KRECs was promptly diagnosed with XLA, the second was the case reported below in this study, and the other ones were false positives with normal number of CD19+ cells at flow cytometric assessment.

At present, specificity and cost-effectiveness are the main concerns regarding its utilization in NBS projects. The risk of high recall rates for PIDs assessment must be evaluated in consideration of parental anxiety and follow-up costs of mild undefined immunological conditions. On the other hand, though requiring long follow-ups, early identification of patients with late-onset immunodeficiency would be an undeniable advantage as it would prevent permanent damage due to recurrent infections in pre-diagnosis period.

TREC use for newborn screening (NBS) of SCID is well known,³ while KREC's potentialities are still to be determined on perspective studies in large cohorts of patients. The following is a case of late presentation of KREC and B cells identified by the screening.

The forward, reverse primers and probes for TREC were obtained from the literature;⁴ the ones for KRECs were designed in our laboratory using Primer Express software version 3.0 (Applied Biosystems). Primer specificity was assessed by BLAST search (<http://www.ncbi.nlm.nih.gov/blast/>), which confirmed their uniqueness. TREC and KREC levels were normalized per microliter of blood, assuming that the sample contains approximately 3 μ L of whole blood. Calibration curves were generated by means of 10-fold serial dilution of plasmids that contain TREC, KREC, and β -actin sequences. Diagnostic cutoff scores were established at 25 TRECs/ μ L and 10 KRECs/ μ L according to the values reported in literature.⁵

We present the case of a newborn male who came to our attention due to an absence of KRECs evidenced by the NBS program. After the confirmation assay, the family was called for PIDs assessment.

Family history was negative for PIDs recurrence but was positive for autoinflammatory disorders as the paternal grandfather suffered from Familial Mediterranean fever. Prenatal history was significant for late onset of intrauterine growth restriction, which led to induce vaginal birth at 37 weeks of gestation. Apgar score at 5 and 10 minutes was, respectively, 9 and 10, and weight at birth was 2050 g. Neonatal course was normal so that the baby was discharged at 3 days after birth with no prolonged stay in a nosocomial environment. Breastfeeding started physiologically and never suffered discontinuance. Physical examination did not detect any anomaly. No drug had been taken both by mother during pregnancy or by the baby at birth, and inflammation markers were negative.

Laboratory tests on peripheral blood showed almost complete absence of CD19+ cells (0.2%; 4.4cell/ μ L), normal values of IgG (935 mg/dL), and undetectable IgA and IgM. Normal values of other lymphocyte subsets ruled out the presence of circulating immature precursors of B cells. Blood group was 0 as that of the mother.

Peripheral T cells and TRECs levels were normal, and CFSE assay showed normal proliferation response to mitogens (IL-2 and PHA).

Suspecting XLA, btk protein was sought and detected on monocytes using flow cytometry (CD45PerCP 5.5, CD14 APC-CY7, CD3 Pe-CY7, CD19 FITC, PE-anti-btk monoclonal antibodies, Becton Dickinson, CA, USA). Genetic analysis of *BTK* gene was performed to exclude missense mutations that allow expression of normal levels of non-functional btk protein. No mutation was found. Similarly, no mutation was found in a preliminary panel of genes (*ICOS*, *TACI*) associated with common variable immunodeficiency (CVID). As leaky forms of SCID cannot display all of the typical clinical and laboratory features when caused by hypomorphic expression of mutations,⁶ we evaluated a preliminary panel of SCID genes including *RAG1* and *RAG2*. Sequencing of those genes showed no causative mutations. Furthermore, no genomic imbalance was detected by array CGH. Genetic tests for autosomal agammaglobulinemia and other genes associated with CVID and SCID, using SCID-targeted gene sequencing panels, were being planned.

Routine testing was periodically carried out at weekly follow-ups. The hypothesis of a hypogammaglobulinemia depending on insufficient transplacental passage of maternal antibodies was excluded in consideration of the gestational age and the normal values of IgG of the baby and of the mother (1052 mg/dL). Moreover, IgA and IgM that cannot cross placenta were persistently undetectable. Maternal origin of IgG was also suggested by the progressive decrease in their serum concentration being 935 mg/dL at 4 days of life and then 742 mg/dL after 1 week from the first detection and then 587 mg/dL after other 7 days. CD19+ cells and KRECs remained absent. Replacement

Abbreviations: CVID, common variable immunodeficiency; DBS, dried blood spot; IUGR, intrauterine growth restriction; IVIG, intravenous immunoglobulins; KREC, K-deleting recombination excision circle; NBS, newborn blood screening; PID, primary immunodeficiency; SCID, severe combined immunodeficiency; TH1, transitory hypogammaglobulinemia of infancy; TREC, T-cell receptor excision circle; XLA, X-linked agammaglobulinemia.

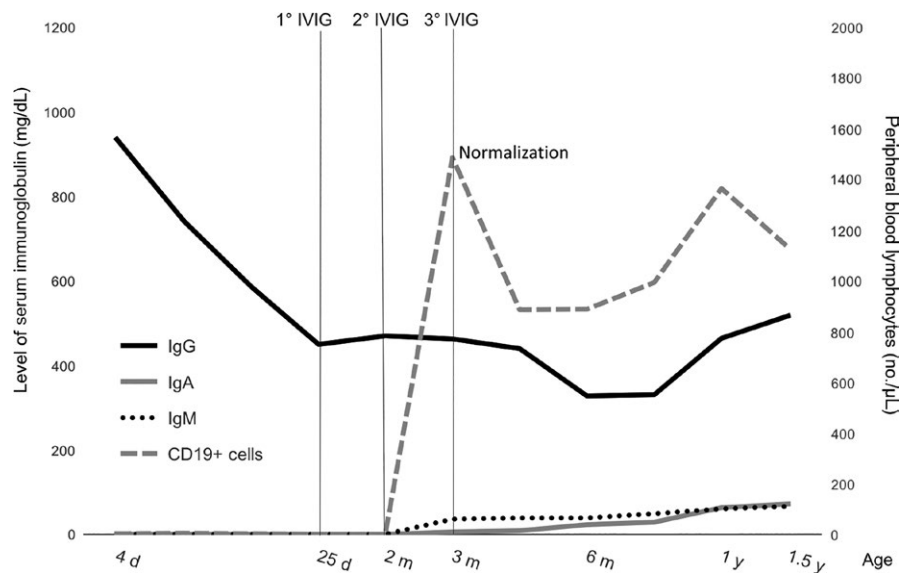


FIGURE 1 Trend of patient's serum immunoglobulins levels and CD19+ cells number in peripheral blood during 1.5 years of life. mo, months; y, years

TABLE 1 Patient's lymphocyte phenotyping at different ages of life and T-cell proliferative response to mitogens performed by FACS. Table also shows serum immunoglobulins levels in several determinations and specific antibody response to T-dependent antigens after complete vaccine schedule of the first year of life

	10 days of life		3 months of life		1 year of life		1.5 years of life	
	%	Cells/ μ L	%	Cells/ μ L	%	Cells/ μ L	%	Cells/ μ L
Cell population								
Lymphocytes		5049		4845		6203		3978
T-lymphocytes	88	4576	64	3129	69	4345	64	2570
CD3+CD4+	74	3848	47	2317	49	3055	43	1744
CD3+CD8+	13	676	16	786	19	1171	17	671
B-lymphocytes	0.1	5	30	1488	22	1366	28	1133
CD19+CD27+					8	110	9	102
CD19+CD27+IgM+IgD+					59	65	64	65
CD19+CD27+IgM-IgD-					41	45	36	37
CD3-CD16+CD56+	9	468	5	228	8	480	7	275
Proliferation assay	%							
CFSE+ w/o stimulus	81%							
PHA (10 μ M)	95%							
Anti-CD3/anti-CD28 beads + rIL-2 (30 U/mL)	85%							
Serum immunoglobulin	mg/dL		mg/dL		mg/dL		mg/dL	
IgG	742		463		464		518	
IgA	UD		7		63.4		73	
IgM	UD		37		61		66	
IgE	UD							
Specific antibodies response								
Tetanus antitoxoid Ab							0.52 IU/mL	
Diphtheria antitoxoid Ab							0.69 IU/mL	
HBS Ab							>1000.00 mUI/mL	

UD, Undetectable.

treatment was therefore started with intravenous immunoglobulins (IVIG) to prevent life-threatening infections.

At the age of 3 months, a sudden recovery of the levels of IgM, IgA, and CD19+ cells was recorded, leading to complete normalization of the values (Figure 1). IVIG treatment and further genetic analysis were suspended for the time being.

After a 1.5-year follow-up, the patient is completely healthy and follow-up visits with laboratory tests for immunological assessment are carried out twice per year. The child has normal levels of immunoglobulin and CD19+ cells; functional B tests (including antitetanus and antihepatitis B antibodies produced after child vaccination) are normal; a low level of memory B cells has been recorded (CD19+ 23%; CD19+CD27+ 8% of CD19+ cells) and is still being monitored (Table 1). Percentage of switched B memory is within the normal range for age (IgD-CD19+CD27+ 41% of CD19+CD27+ cells).

No similar cases have been described so far.

Previous reports and retrospective studies reported rare false-positive diagnoses of SCID for low levels of TREC^{3,7,8} and correlated the absence of TREC to placental pathologies, pre-term birth, and serious infant illnesses.⁹ Very little is known regarding false-positive diagnosis of B-cell defects due to low levels of KREC; however, even assuming that KREC has a similar behavior to TREC, that condition does not apply to our patient. In fact, flow cytometry confirmed that low KREC levels were associated with the absence of CD19+ cells and no production of immunoglobulin by the patient, so that the case cannot be considered as a false positive. KREC is a marker of bone marrow B-cell output and is used in the screening to identify hypogammaglobulinaemia¹⁰ that was actually later confirmed in our patient.

Late production of serum immunoglobulin is a common event, but to our knowledge, this is the first reported case of late CD19+ cells occurrence and subsequent immunoglobulin production, as those cases cannot be identified in the absence of newborn screening. We cannot exclude that a portion of patients with transient hypogammaglobulinemia had reduced or absent B cells at birth but normal values of B cells at the time of diagnosis, months later, and that the absence of B cells has never been identified so far for the absence of a SCID NBS program. However, the clinical history of the described patient is completely different from a THI, as immunoglobulin levels increased at the same time of CD19+ cells and KREC recovery.

Intestinal microbiota alterations are known to cause delays and modifications in antibody production mainly regarding IgA, but in the presented case the major perinatal causes of altered bacterial gut colonization were missing.

At present, we cannot exclude that the clinical history we presented is a paraphysiological condition with no risk for child health but, at the same time, we cannot exclude that the present normalization of immunological parameters is a honeymoon phase in an

immunodeficiency that will reveal itself later in life. Inclusion of similar cases in large multinational registers is necessary to better understand late-onset immunopathological conditions.

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