

Letter to the Editor

Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies

To the Editor:

Familial hemophagocytic lymphohistiocytosis (FHL) is an often-fatal hyperinflammatory disorder caused by mutations in different genes.¹ It can be diagnosed on the basis of a family history of hemophagocytic lymphohistiocytosis, by genetic testing and findings of defective lymphocyte cytotoxicity.² A rapid diagnosis is crucial to administer effective first-line treatment, to determine the need for hematopoietic stem cell transplantation (HSCT), and to reduce possible post-HSCT sequelae.² Given the estimated incidence of 1/50,000 live births, the strong impetus for early diagnostics, and the life-saving curative nature of HSCT in FHL, the disease is a candidate for population-scale newborn screening.^{3,4} High-throughput assays that can identify severe combined immunodeficiencies (SCIDs) and primary agammaglobulinemias (eg, X-linked agammaglobulinemia) through simultaneous quantification of T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) in dried blood spot samples (DBSSs) represent first examples of effective neonatal screening for severe primary immunodeficiencies (PIDs).⁵ Newborn screening assays have not been developed for FHL because of the large diversity of mutations not amenable to rapid screening tests. However, we recently identified an inversion straddling the *UNC13D* locus as a frequent cause of FHL in Scandinavia.⁶ In Sweden, approximately 50% of infant patients with FHL are homozygous for this aberration, providing a candidate gene for neonatal screening and illustrating how genetic screening may be customized to indigenous immunogenetic disease predispositions.

For detailed information on experimental methods, see this article's [Methods](#) section in the Online Repository at www.jacionline.org.

We developed a newborn screening assay for patients homozygous for the *UNC13D* inversion (*UNC13D*^{inv/inv}). This triplex assay combines the measurement of TREC and KREC copy numbers in DBSS with a newly introduced quantification of *UNC13D*^{wt} copies in exchange for beta-actin (*ACTB*), which previously served as a control of DNA integrity.⁵

We screened for the lack of *UNC13D*^{wt} copies as a feature of *UNC13D*^{inv/inv} patients with FHL, yet heterozygous carriers might inadvertently stand out in quantitative PCR (qPCR) analyses because of the inherent genotypic reduction of the template. Thus, we applied a dual-detection-probe approach targeting a polymorphic region in *UNC13D* (see [Fig E1](#) in this article's Online Repository at www.jacionline.org), thus introducing analytical noise able to mask *UNC13D*^{inv} carriers (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Of selected stored, original Guthrie cards, all *UNC13D*^{inv/inv} patients with FHL were correctly identified as lacking *UNC13D*^{wt} copies (termed "positive" screening result), whereas none of the carriers was picked up ([Fig 1, A](#); and see [Fig E3](#) in this article's Online Repository at www.jacionline.org). In a screen of deidentified 2763 DBSSs, an arbitrary cutoff of 100 *UNC13D*^{wt} copies was set, resulting in retesting of

63 newborn samples initially detected with a low number of *UNC13D*^{wt} copies ([Fig E3](#)). Reevaluation of these samples revealed that the initial low number of *UNC13D*^{wt} copies was mostly due to insufficient DNA yield (low *ACTB* values) as a result of the punching position in 53 individuals. Of note, *UNC13D*^{wt} copies could generally be used to verify DNA integrity for the TREC-KREC assay analogous to *ACTB* quantification (see [Fig E4, A](#), in this article's Online Repository at www.jacionline.org).

A second-tier multiplex PCR analysis step subsequent to the TREC-KREC-*UNC13D*^{wt} screening assay was introduced (see [Fig E5](#) in this article's Online Repository at www.jacionline.org) to limit the number of newborns referred for functional third-tier evaluation. Of 10 samples with repeatedly low number of *UNC13D*^{wt} copies in the qPCR analyses, 8 turned out normal by multiplex PCR analysis. Thus, 2 of 2763 anonymous newborns (0.07%) had a positive screening result ([Fig E3](#)), which is an acceptable recall rate considering existing newborn screening assays. Because of the anonymous nature of the DBSS, no further clinical investigation was done and it remains to be determined whether rare polymorphisms may have affected primer/probe hybridization in these 2 individuals. A recall rate of 0.07% for functional analysis is regarded as acceptable, but it may be further reduced by genetic insights gained from the screening of larger cohorts. Evaluation of CD107a surface expression on cytotoxic lymphocyte stimulation represents a robust third-tier confirmatory test.⁷

None of the patients with FHL or carriers displayed pathologically low TREC or KREC copy numbers ([Fig 1, B](#)), and all SCID and X-linked agammaglobulinemia control samples showed normal *UNC13D*^{wt} copy numbers ([Fig 1, A](#)). Remarkably, the *UNC13D*^{inv/inv} patients displayed a significantly reduced number of KREC copies ($P = .011$; Mann-Whitney U test), indicating that deviations in the B-cell compartment are present from birth in these patients. Aberrations in humoral immunity and B-cell phenotype have been described in patients with FHL, most commonly following EBV-induced inflammation.⁸ However, hypogammaglobulinemia has also been described in patients with FHL before disease presentation,^{8,9} possibly suggesting defects in B-cell differentiation. Our observation of decreased signal-joint KREC numbers in *UNC13D*^{inv/inv} patients rather suggests an early impairment of B-cell development in the bone marrow. Remarkably, IFN- γ -mediated suppression of B-cell development has been reported in mice.¹⁰ Correlation analysis of TREC and KREC copy numbers, measured by triplex qPCR with either *ACTB* or *UNC13D*^{wt} probes, indicated comparable performance between the 2 assays ([Fig E4, B](#) and [C](#)). Thus, *UNC13D*^{wt} data can support the interpretation of DNA integrity in the newborn screening of severe T- and B-cell lymphopenias.

In summary, we have developed and successfully evaluated a combined newborn screening assay for an FHL3 aberration endemic to Scandinavia, SCID, and primary agammaglobulinemias. Merging 3 disease screening markers in 1 assay is likely to further improve the cost-efficacy of neonatal screening programs for PID.¹¹ With current technology, DBSS-based assays for all FHL mutations are inconceivable. Still, other examples of singular mutations being frequent causes exist,¹²

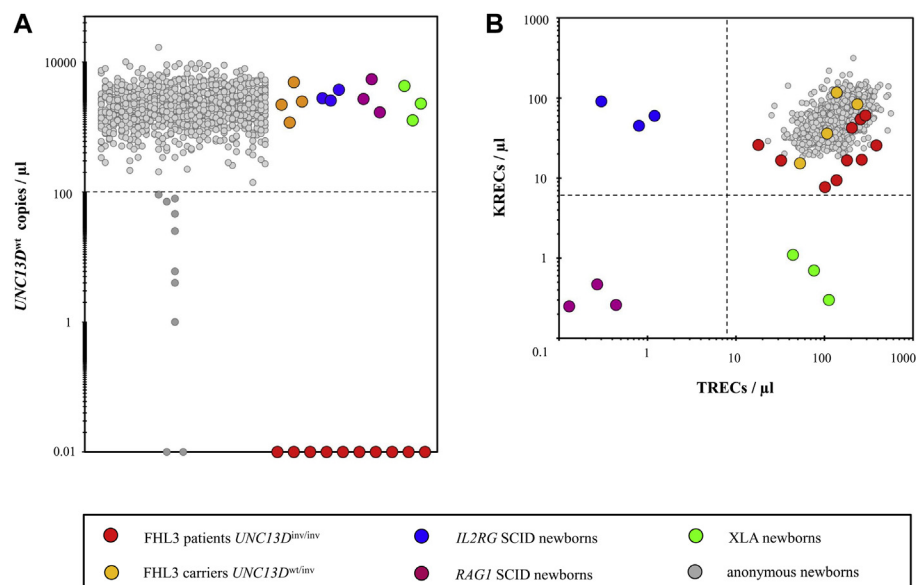


FIG 1. Quantification of *UNC13D*^{wt} (**A**) and TREC and KREC (**B**) copy numbers in DBSS from patients with FHL, carriers, anonymous newborns, and PID patients with SCID or XLA. The arbitrary cutoff for *UNC13D*^{wt} copies is depicted as a *dashed line*. Abnormal ("positive") screening results were defined as less than 8 TRECs/ μ L and less than 6 KRECs/ μ L of dried blood, according to current prospective screening trials in Sweden and Germany. *inv*, Inversion; *wt*, wild type; *XLA*, X-linked agammaglobulinemia.

thus representing potential targets for neonatal screening programs.

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METHODS

Preparation of DNA from patients and anonymous newborns

Single 3.2-mm DBSS punches were obtained from original Guthrie cards of 10 patients with *UNC13D*^{inv/inv} FHL, 4 individuals with *UNC13D*^{wt/inv}, and 2763 randomly selected anonymous Swedish newborns. In addition, DBSSs from patients with SCID (*IL2RG* or *RAG1* mutations) or agammaglobulinemia (*BTK* mutations) were used as reference controls for the TREC/KREC screening assay. The clinical phenotype and laboratory findings of the included patients with FHL have been reported previously.^{E1} DNA was extracted from DBSS as previously described.^{E2} This study was approved by the ethics committee at Karolinska Institutet, and written parental consent was obtained for all included patients and carriers.

qPCR screening assay, *UNC13D* plasmid standard, and multiplex PCR analysis

qPCR for TRECs, KRECs, and beta-actin (*ACTB*) was performed on a ViiA7 real-time PCR system (Applied Biosystems, Foster City, Calif) as previously described.^{E2} Of note, the KREC assay detected the KREC signal-joint.^{E3} The qPCR procedure was optimized on the basis of custom reagents provided by Affymetrix (Santa Clara, Calif). For detection of *UNC13D*^{wt} copies, the *ACTB* probe and primers were replaced by 2 hydrolysis probes and primers directed against the *UNC13D* wild-type sequence (Table E1 and Fig E1).

The plasmid standard for absolute quantitation of *UNC13D*^{wt} copies was generated from amplified human whole-blood DNA, subcloned into the pCR 2.1-TOPO vector (Life Technologies, Carlsbad, Calif), using primers detailed in Table E1.

Multiplex PCR of DNA samples from peripheral blood and DBSS eluates was performed with primers detailed in Table E1. Separated PCR products were analyzed using DNA microfluidic-chips on a 2100 Bioanalyzer (both Agilent Technologies, Santa Clara, Calif).

Statistical analysis

Comparison in between groups and correlation analysis were performed using XLSTAT 2013 (Addinsoft, Paris, France). Statistical significance was defined as $P < .05$.

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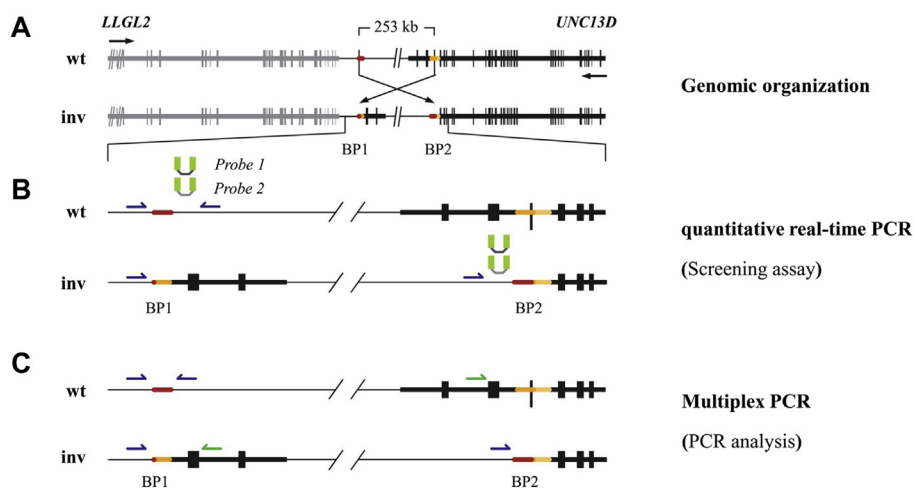


FIG E1. Schematic illustration of *UNC13D* wild-type (*wt*) and inversion (*inv*) alleles adapted from Meeths et al.⁶ **A**, The breakpoints (BP) are located in Alu elements containing an identical sequence of 25 bp. Two elements, AluSc8 and AluSx1 (yellow, orange), are located on the reverse strand in intron 30 of *UNC13D*, and an AluY (red) element is located 253 kb upstream of *UNC13D* on the forward strand. **B**, Position of primers and hydrolysis probes for the detection of *UNC13D*^{wt} copy numbers. In the qPCR screening assay, the primers do not amplify the *UNC13D*^{inv} allele. **C**, Position of primers used in the multiplex PCR analysis step for the amplification of both the *UNC13D*^{wt} and the *UNC13D*^{inv} allele.

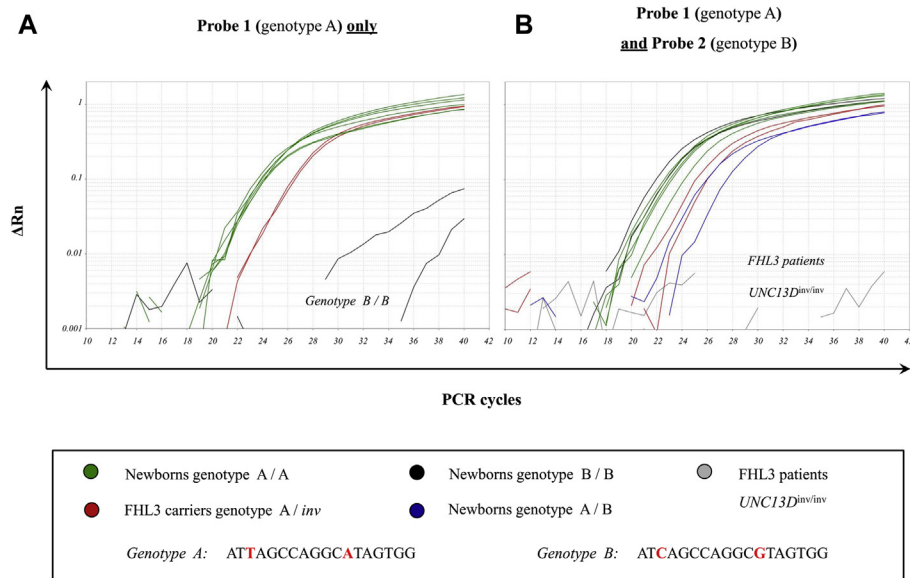


FIG E2. qPCR amplification plots for the *UNC13D^{wt}* hydrolysis probes targeting either genotype A only, (A) or genotype A and B (B) of the polymorphic probe hybridization site. *inv*, Inversion; *wt*, wild type.

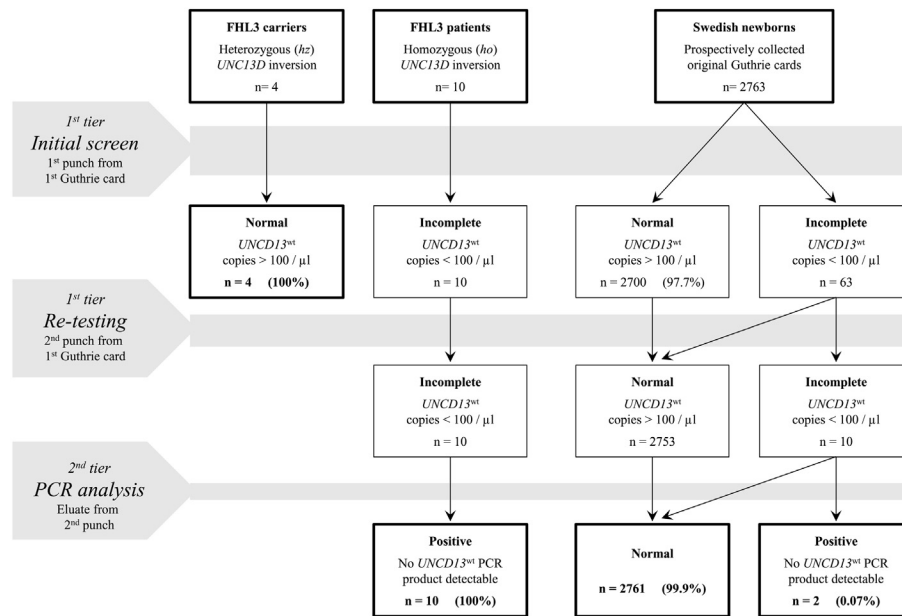


FIG E3. Flow chart of the TREC-KREC-*UNC13D*^{wt} qPCR dried blood spot screening assay and the multiplex PCR analysis step. *wt*, Wild type.

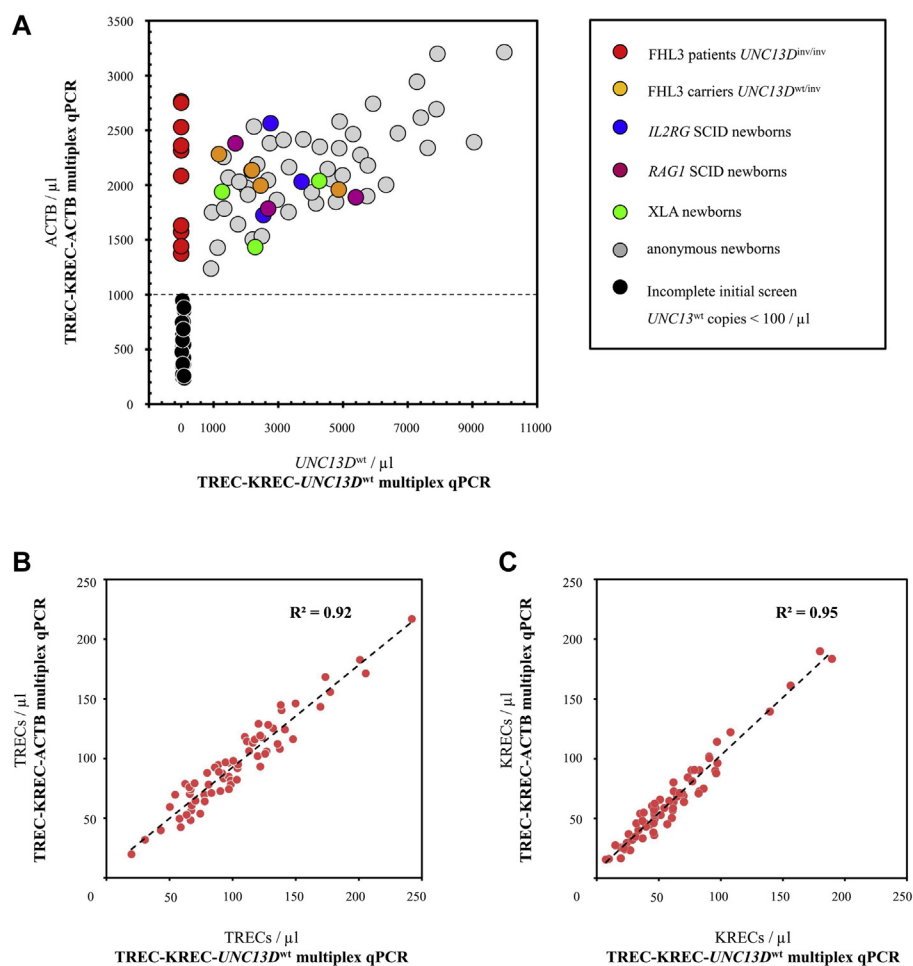


FIG E4. A, Comparison of $ACTB$ and $UNC13D^{wt}$ copy numbers in incomplete samples on initial screen, patients with FHL and carriers, patients with PID with SCID or XLA, and anonymous newborns. Correlation analysis of TREC copy numbers (B) or KREC copy numbers (C) measured by triplex qPCR with either $ACTB$ or $UNC13D^{wt}$ probes. XLA, X-Linked agammaglobulinemia; wt, wild type.

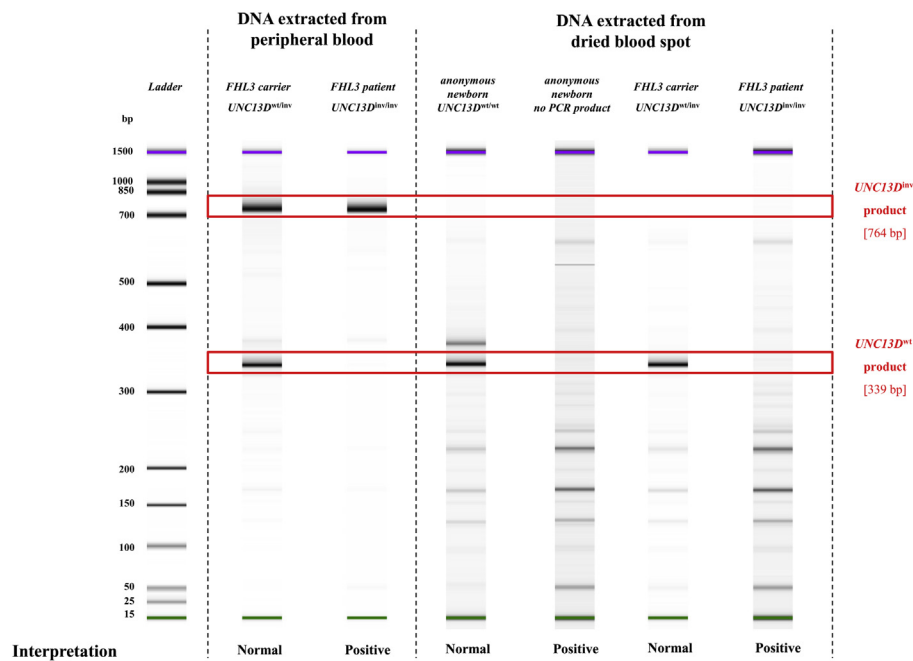


FIG E5. Product analysis of the multiplex PCR for the detection of *UNC13D^{wt}* and *UNC13D^{inv}* alleles with DNA samples from peripheral blood or DBSS eluates. “Normal” and “positive” screening results from the second-tier multiplex PCR indicated below the lanes. *inv*, Inversion; *wt*, wild type.

TABLE E1. Primer and probe oligonucleotide sequences used for qPCR, plasmid preparation, and multiplex PCR

Primer/probe sequence
UNC13D _{wt} real-time PCR screening assay
Probe 1: 6-FAM-ATTAGCCAGGCATAGTGG
Probe 2: 6-FAM-ATCAGCCAGGCGTAGTGG
Forward primer: TGTCGGTGTGCATATTCAG
Reverse primer: TGGGTTCACCCCACTGTC
UNC13D _{wt} plasmid preparation
Forward primer: TGCTGAACTTTGAGCTTTGC
Reverse primer: AGAGAGGAAGATGCCACGTC
UNC13D multiplex PCR analysis
Reverse primer wt: CGTAGTCCAGCACGGTGAG
Forward primer inv: TGCTGAACTTTGAGCTTTGC
Forward primer wt: AGTGGGGTGGCATGATCTC

inv, Inversion; *wt*, wild type.