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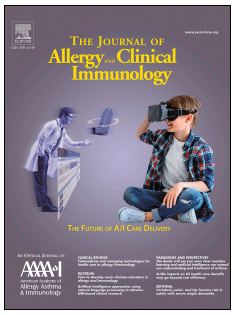

Genetic errors of immunity distinguish pediatric non-malignant lymphoproliferative disorders

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Genetic errors of immunity distinguish pediatric non-malignant lymphoproliferative disorders

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Abstract

Background: Pediatric non-malignant lymphoproliferative disorders (PLPD) are clinically and genetically heterogeneous. Long-standing immune dysregulation and lymphoproliferation in children may be life-threatening, and a paucity of data exists to guide evaluation and treatment of children with PLPD.

Objective: The primary objective of this study was to ascertain the spectrum of genomic immunologic defects in PLPD. Secondary objectives included characterization of clinical outcomes and associations between genetic diagnoses and those outcomes.

Methods: PLPD was defined by persistent lymphadenopathy, lymph organ involvement, or lymphocytic infiltration for more than 3 months, with or without chronic or significant EBV infection. Fifty-one subjects from 47 different families with PLPD were analyzed using whole exome sequencing (WES).

Results: WES identified likely genetic errors of immunity in 51% to 62% of families (53% to 65% of affected children). Presence of a genetic etiology was associated with younger age and hemophagocytic lymphohistiocytosis. Ten-year survival for the cohort was 72.4%, and patients with viable genetic diagnoses had a higher survival rate (82%) compared to children without a genetic explanation (48%, $p = 0.03$). Survival outcomes for individuals with EBV-associated disease and no genetic explanation were particularly worse than outcomes for subjects with EBV-associated disease and a genetic explanation (17% vs. 90%; p =

0.002). Ascertainment of a molecular diagnosis provided targetable treatment options for up to 18 individuals and led to active management changes for 12 patients.

Conclusion: PLPD therefore defines children with high risk for mortality, and WES informs clinical risks and therapeutic opportunities for this diagnosis.

Clinical Implications

Genetic evaluation is necessary in PLPD because it not only helps to determine the underlying mechanistic etiology of disease and carries prognostic implications, but it also directs key management decisions.

Capsule Summary

Genetic errors of immunity are prevalent in children who meet criteria for PLPD yet correlate with improved survival. EBV-PLPD without a genetic explanation is associated with increased risk for mortality. Genetic testing alters management strategies.

Key Words: lymphoproliferation**,** pediatric**,** whole exome sequencing, genomic, Epstein-Barr virus

Abbreviations

ALPS - autoimmune lymphoproliferative syndrome

CAEBV - chronic active EBV

CMG - Center for Mendelian Genomics

EBV - Epstein-Barr virus

EBV-PLPD - EBV-associated PLPD

HGSC - Human Genome Sequencing Center

HLH - hemophagocytic lymphohistiocytosis

HSCT - hematopoietic stem cell transplantation

IUIS - International Union of Immunological Societies

NK - natural killer

PIDD - primary immunodeficiency disease

PIRD - primary immune regulatory disorder

PLPD - pediatric non-malignant pediatric lymphoproliferative disorders

WES - whole exome sequencing

Introduction

Lymphadenopathy is common during normal childhood and noted on physical examination of approximately half of all children visiting a medical provider for either “well” or “sick” visits.[1] While transient lymphadenopathy in children is rarely dangerous, long-standing lymphoproliferation may reflect underlying immune dysregulation, increase the risk for developing malignant disease or hemophagocytic lymphohistiocytosis (HLH), and/or drive life-threatening lymphoproliferative disease.[1-3]

Non-malignant pediatric lymphoproliferative disorders (PLPD) constitute a clinically and genetically heterogeneous group of conditions associated with a wide range of clinical consequences. PLPD are characterized by proliferating (and/or persistent) clonal or polyclonal lymphoid cells that may arise as aberrant responses to immune stimuli or represent intrinsic immune dysregulation.[4] Clinical presentations include chronic or recurrent lymphadenopathy, splenomegaly, or symptoms secondary to organ infiltration by abnormal lymphoid cells. In some cases, patients may develop pathologic inflammation consistent with HLH or macrophage activation syndrome**.** PLPD are also associated with an increased predisposition toward developing hematopoietic malignancies, specifically lymphoma.[5-7] When a lymph node biopsy is negative for malignancy, the diagnostic and therapeutic paths forward for children with evidence of lymphoproliferation remain poorly defined.

Although several inherited diseases of immune dysregulation have been associated with PLPD, the frequency and distribution of primary immunodeficiency diseases (PIDD) and primary immune regulatory disorders (PIRD) in children with PLPD are unknown. PIRDs encompass immune mediated disease leading to autoimmune disease and autoinflammatory conditions[8, 9]. Errors in more than 400 genes are now ascribed to PIDD and PIRD[2, 8], and a significant number of these conditions present with clinical features consistent with PLPD.

PLPD associated with Epstein-Barr virus (EBV) can represent *de novo* infection, reactivation, and/or malignant transformation[7, 10]. PIDD patients who have impaired natural killer (NK) cell cytotoxic function may have increased susceptibility to primary infection or reactivation of viruses, including EBV[11]. Patients with chronic active EBV (CAEBV), a rare form of EBV disease characterized by persistent and/or proliferative EBV-infected lymphocytes during primary or reactivated EBV infection[12], have poor outcomes, especially individuals with EBV specifically detected in NK and T cells[12, 13].

Optimal management of PLPD patients requires understanding of underlying pathogenic drivers. Given the rare occurrence of PLPD and its overlapping features with ordinary reactive lymphadenopathy in children, diagnosis is often quite challenging. We therefore sought to determine the utility of whole exome sequencing (WES) in children with PLPD with a focus on impact on treatment and prognosis.

Methods

Subject Enrollment

Patients and family members at Texas Children's Hospital or collaborating referral centers who met criteria for PLPD between 1994 to 2018 were offered participation in this study. Studies were performed under research protocols approved by the Baylor College of Medicine Institutional Review Board. All procedures involving human participants were performed in accordance with institutional and international ethical standards.

Clinical Data and Study Criteria

"PLPD" was defined as persistent lymphadenopathy, lymph organ involvement, or organ lymphocytic infiltration with duration greater than 3 months, with or without chronic or significant EBV infection in children and young adults (≤21 years). Chronic or significant EBV infection was defined by recurrent or persistent EBV viremia greater than 3 months, invasive EBV disease, or EBV DNA copy number >100,000 in either whole blood or plasma[13, 14]. Exclusion criteria consisted of history of hematopoietic cell transplantation, solid organ transplantation, established diagnosis of autoimmune lymphoproliferative syndrome (ALPS), or malignancy prior to PLPD. Biopsy details are included in **Supplemental File: Master Data Table.** Data regarding co-morbidities and clinical outcomes were extracted from the medical record.

Whole Exome Sequencing and Data Analysis

Clinical whole exome sequencing was conducted by Baylor Genetics Laboratories (Houston, TX, USA). Research-based WES was performed at the Human Genome Sequencing Center (HGSC) at Baylor College of Medicine through the Baylor-Hopkins Center for Mendelian Genomics (CMG) initiative. Using 1 μg of DNA, an Illumina paired-end pre-capture library was constructed according to the manufacturer's protocol (Illumina Multiplexing_SamplePrep_Guide_1005361_D) with modifications as described in the *BCM-HGSC Illumina Barcoded Paired-End Capture Library Preparation* protocol. Pre-capture libraries were pooled into 4-plex library pools and then hybridized in solution to the HGSC-designed Core capture reagent[15] (52 Mb, NimbleGen), or 6-plex library pools used the custom VCRome 2.1 capture reagent[15] (42 Mb, NimbleGen) according to the manufacturer's protocol (*NimbleGen SeqCap EZ Exome Library SR User's Guide*) with minor revisions. The sequencing run was performed in paired-end mode using the Illumina HiSeq 2000 platform, with sequencing-by-synthesis reactions extended for 101 cycles from each end and an additional 7 cycles for the index read. With a sequencing yield of 9.1 Gb, the sample achieved 91% of the targeted exome bases covered to a depth of 20X or greater. Illumina sequence analysis was performed using the HGSC Mercury analysis pipeline (https://www.hgsc.bcm.edu/software/mercury)[16, 17], which moves data through various analysis tools from the initial sequence generation on the instrument to annotated variant calls (SNPs and intra-read in/dels). Data were analyzed through the Baylor-Hopkins CMG initiative from 2015 to 2019, as previously described.[18, 19] Variants were prioritized according to established guidelines[20, 21]

with additional attention to variants in genes established by the International Union of Immunological Societies (IUIS)[2, 8] to be defective in human immunologic disorders or closely associated with these genes in known protein interactions or immunologic pathways (**Table S1**). Genetic variants were ultimately assigned to the following categories describing potential contributions to immune pathogenesis: 1) defective control of lymphocyte activity; 2) impaired activation/cytotoxicity, cytoskeletal organization and apoptosis; and 3) dysregulated inflammation.

Statistical Analysis

Demographic and clinical information were abstracted from medical records. The chi-squared test was used if counts exceeded $n = 5$; otherwise Fisher's Exact test was implemented. Kaplan-Meier survival curves were generated to estimate survival from time of disease presentation to end of follow-up, and a log-rank test estimated differences across strata of interest. All statistical analyses were conducted in STATA 13.v1.

Results

Characteristics of PLPD Cohort

Clinical Features

Overall, 51 subjects from 47 families met criteria for PLPD at Texas Children's Hospital and referring centers (**Table 1**). The median age at disease presentation was 3.3 years (range 4 weeks – 21 years) with nearly equal proportions of males (*n* = 26) and females (*n* = 25). Almost half (49%) of subjects were Hispanic, and 29% were non-Hispanic white. All patients met at least one PLPD criterion: 38 patients (74%) had lymphadenopathy for longer than 3 months, 32 patients (63%) had splenomegaly, and 12 patients (23%) had non-malignant lymphoproliferation on tissue biopsy. Therapeutic strategies ranged from observation to hematopoietic stem cell transplantation (HSCT). Maximum interventions in ascending order included observation (21.6%), steroids only (15.7%), biologics (19.6%), chemotherapy (21.6%), and HSCT (15.7%).

Hemophagocytic lymphohistiocytosis and EBV

Among the 51 subjects, 15 patients (29%) fulfilled at least five of eight HLH-2004[22] diagnostic criteria for HLH: 9 (60%) survived, and 8 (53%) had EBV-associated disease (**Table 1, Table S2**). Among the entire cohort, 21 (41%) had EBV-PLPD and 14 (67%) of these patients survived (**Table 1, Table S3**). Five of 8 (63%) patients with both EBV-PLPD and HLH survived, and 9 of 12 (75%) patients with EBV-PLPD without HLH survived.

Autoimmune and Autoinflammatory Conditions

Fifteen subjects (29%) were diagnosed with autoimmune and/or autoinflammatory conditions either prior to or concurrent with their PLPD diagnosis (**Table 1, Table S4**), and this subset of patients had an overall survival rate of 73%. Of the 22 subjects who had testing for double negative alpha-beta T cells, 11 had elevated levels (≥ 1.5% of total lymphocytes). ALPS was considered at some point in the medical record in 40 patients (78%), but upon evaluation none in this cohort met diagnostic criteria[23, 24] prior to enrollment, and no functional defects in apoptosis were identified. However, ALPS-associated gene defects were subsequently identified in 2 patients in whom ALPS was not initially suspected or evaluated. For reference, 14 patients were diagnosed with ALPS at our institution during the study period (and were therefore excluded from this cohort).

Malignancy

Subjects with lymphoproliferative disease secondary to malignancy were excluded from this study (**Table 1, Table S5**). Four patients (8%) developed malignancy after meeting enrollment criteria for non-malignant PLPD. Median time interval between PLPD presentation and malignancy diagnosis was 7.75 years (**Table S5**). All of these patients (100%) initially had EBV-associated PLPD with subsequent diagnosis of either mature T-cell lymphoma (*n* = 1), diffuse large B cell lymphoma (*n* = 2), or papillary thyroid carcinoma (*n* = 1). Notably, only the patient with papillary thyroid carcinoma, which is not typically associated with

lymphoproliferative disease, EBV infection, or immune deficiency, survived (25%).

Genetic Findings

Genetic Errors of Immunity are Prevalent in PLPD

All 51 participants from the 47 families underwent WES. Clinical WES was completed in 19 of the families (19 probands), resulting in genetic diagnoses for only 4 children (21%). For the other 15 cases and families who underwent clinical WES which did not yield a diagnosis, 12 consented to research-level analyses of the clinical exome data, resulting in identification of an additional 8 candidate molecular diagnoses. For one of these families, research WES of 2 additional affected siblings enabled identification of the defect in *PIK3CD* in all 3 children. Research-based WES analyses were also performed without clinical WES for 28 families (30 cases), leading to likely molecular diagnoses in 13 (46%) [14 cases, 47%] and further potential genetic explanations in 4 (14%) [5 cases, 17%]. Thus, 29 of 47 PLPD families (62%), or 33 of 51 affected children (65%), were found to have likely or plausible disease-associated genetic errors of immunity (**Table S1**). Note that "genetic errors" serves as a more appropriate term than "inborn errors" because of the identified likely somatic changes to *KRAS* and *NRAS.* Of these 29 families (33 cases) with viable genetic explanations, 21 (23 cases) had disease candidate variants in 15 IUIS-established PIDD and PIRD genes[2, 3, 8]. One family (LPD019 and LPD034) was discovered to have a novel disease candidate for which the variants (in *NCKAP1L*) were functionally validated[25]. In the

232 remaining 7 families (8 cases) with genetic disease candidates, one was hypoth-
233 esized to have phenotypic expansion of a known disease-associated gene
234 (*CDC42*[26, 27]), and 6 (7 cases) had potentially novel genetic causes of human
235 disease. At minimum, 24 of 47 families (51%), or 27 of 51 affected children
236 (53%), had pathogenic or likely pathogenic genetic etiologies for LPD. A smaller
237 proportion of patients (21%) who received only clinical WES resulted in like-
238 ly/potential diagnoses versus 61% who underwent research WES only (p = 0.01).
239 Further, when considering children who underwent clinical WES followed by re-
240 search-based analysis, 63% obtained likely/potential diagnoses, compared to on-
241 ly 21% who had clinical WES only (p = 0.003). Rather than suggesting inferiority
242 of clinical testing, these observations reflect the improvement in WES methodol-
243 ogy over the course of the study period. All of the LPD-associated genes were
244 observed to fall broadly into one of 3 categories[2, 3] based on immunologic mech-
245 anism: 1) defective control of lymphocyte activity; 2) impaired lymphocyte activa-
246 tion/cytotoxicity, cytoskeletal organization, and apoptosis; and 3) dysregulated
247 inflammation (**Figure 1**).
248
249 *Genotype/Phenotype Correlations*
250 The proportion of subjects with a potential molecular explanation inversely corre-
251 lated with age at presentation (**Figure 2A**). Patients with suggested genetic ab-
252 normalities were significantly younger at presentation compared to subjects who
253 lacked genetic findings (p = 0.02, **Figure S1**). In fact, all children (n = 7, 100%)
254 who presented with PLPD younger than one year old were found to have a viable

genetic explanation for the disease. Of the 28 patients between 1 and 8 years of age, 72% had a potential genetic etiology identified. In contrast, a molecular diagnosis for PLPD was less likely to be identified in the 16 patients who developed symptoms after 8 years of age (38%).

The proportion of patients with possible genetic explanations did not differ significantly between EBV-PLPD and PLPD without EBV. Of the 21 patients with EBV-PLPD, 67% had potential genetic explanations, and of the 27 patients with PLPD without EBV, 70% had implicated genetic findings (p = 0.91). Likewise, among the three immune-mediated genetic categories, the proportion of EBV-affected individuals was evenly distributed (**Figure 1**).

Genetic findings were more common in patients with HLH compared to patients who eventually developed malignancy, although the proportional differences did not reach a level of statistical significance (p = 0.08). Among the 15 patients who met HLH diagnostic criteria[13, 22], a probable genetic explanation was present in 11 (73%), 9 of whom were under the age of 8 (**Table S2**). Fewer patients who developed malignancy subsequent to their PLPD diagnosis (25%) had a genetic disorder (**Table S5**).

Lack of Genetic Diagnosis is Associated with Increased Risk for Mortality

Estimated ten-year survival for the entire cohort was 72.4% with a median follow-up of 5.6 years (range 0.10 - 26.6 years, **Figure 2B**). Analyzing the cohort as a

whole (**Figure 2C, Figure S2**), patients without an identified possible genetic etiology had significantly lower ten-year survival compared to patients with a potential genetic explanation (48% versus 82%, respectively, $p = 0.03$). The ten-year survival estimate for children with EBV-PLPD trended lower compared to children without EBV (56% vs 80%; $p = 0.13$). Children with EBV-PLPD frequently had complicated courses: 5 had HLH, 4 developed malignancy, and 1 developed both malignancy and HLH. Presence of EBV-PLPD did not predict an underlying genetic defect. Most notably, however, subjects with EBV-PLPD without a viable genetic explanation had significantly lower estimated survival than children with a suggested genetic explanation (17% vs. 90%, $p = 0.002$; **Figure 2D**). In fact, the group of patients who had EBV-PLPD without a genetic explanation was the category associated with the highest risk of death.

Genetic Testing Impacts Therapeutic Decisions

Identification of an underlying genetic diagnosis in PLPD patients informs therapeutic opportunities (**Figure 4, Table S6**). Currently, targeted therapies are available or show promise for treatment of at least 11 of the genetic conditions diagnosed in this cohort (potentially benefitting up to 18 patients from 16 families)[28]. Furthermore, successful outcomes have been reported after HSCT in 10 of the 15 IUIS-recognized genetic errors of immunity reported here (which could treat up to 20 patients from 18 families). Prior to the availability of genetic testing results, only two patients had received empiric treatment that would have been supported by their ultimate genetic diagnoses. After genetic testing results were

available, 12 patients had diagnoses that led to active changes in the treatment plan through either targeted therapies or planning for HSCT. Five patients who had actionable findings after genetic testing did not have changes in their treatment plans, as they were either clinically well or lost to follow-up. Unfortunately, three patients died prior to receiving their genetic diagnoses (*NRAS, KRAS,* and *CASP1*). Importantly, 6 novel disease candidate genes were discovered, which may lead to unique opportunities for precision therapy. It becomes important to note that estimated ten-year survival was greatest (100%, *n* = 10) among subjects in whom control of disease was achieved using targeted biologic therapies (**Figure S3**).

Discussion

Clinical and Genomic Landscape of PLPD

Pediatric non-malignant LPD represents a heterogeneous group of conditions with high risk for mortality characterized by lymphadenopathy and/or lymph organ involvement with or without chronic, severe, or recurrent EBV infection. HLH has been associated with a range of lymphoproliferative disorders[29, 30] and was enriched in this cohort, with 15 (29%) of 51 children meeting HLH-2004 diagnostic criteria. Children with immune disorders also carry increased risk of malignancy[31]. Despite exclusion of malignancy at presentation, 8% of this PLPD cohort subsequently developed this complication.

In order to improve knowledge of underlying immune pathogenesis mechanisms in PLPD to better inform treatment, we performed WES of 51 subjects from the 47 families in this cohort. This unbiased approach led to a genetic diagnosis in 51% to 62% of families [53% to 65% of affected children] (**Figure 1**), encapsulating a heterogenous collection of genetic errors of immunity. As a comparison, Stray-Pedersen et al reported a 40% overall genetic diagnostic rate, including potentially novel diseases, in patients with PIDD.[18] Findings from this study support the clinical utility of comprehensive genetic analysis in PLPD, with high likelihood of identifying genetic alterations that inform therapeutic opportunities and clinical risk.

PLPD Risk Stratification

Overall survival was 72% with a trend towards worse outcomes associated with EBV infection, HLH, and subsequent malignancy. Earlier age at presentation with LPD positively correlated with likelihood of identifying a potential genetic diagnosis, especially in children with impaired lymphocyte activation/cytotoxicity, cytoskeletal organization, and apoptosis (**Table S7**). In fact, a molecular explanation was found in all 7 patients who presented at less than 1 year of age. These data particularly support the clinical utility of WES for infants and younger children with PLPD. At older ages, acquired factors, such as autoimmune disease and infection, may also contribute to development of PLPD. Even so, for 9 patients above 12 years of age, 3 had a plausible underlying genetic explanation, suggesting that genetic testing can play a critical role in diagnosis and management of PLPD in adolescents and young adults as well.

Increased Mortality in Patients with EBV-PLPD and No Genetic Explanation

EBV is the most common pathogen associated with non-malignant LPD[32]. In this cohort, patients with EBV-PLPD had pathogenic or likely pathogenic variants in several genes associated with atypical EBV disease: *CTLA4, LRBA, PIK3CD, CD27, RAB27A, ZBTB24,* and *STAT1*[33]. Additionally, somatic *PLCG2* mutations have correlated with EBV-positive Burkitt lymphoma[34]. Potentially disease-associated variants in *CASP1* and *CASP5* were also discovered in EBV-PLPD patients [24, 35-46]. CASP1 has provocatively been implicated in IRF8-dependent EBV lytic reactivation[47]. EBV status alone, however, did not impact the likelihood of having a potential underlying genetic explanation for LPD (67% of EBV-

associated LPD vs. 70% of non-EBV-associated LPD). Furthermore, susceptibility to EBV infection was not significantly skewed toward any of the three immunologic mechanism categories (**Figure 1**). However, children with EBV-associated PLPD without an identifiable genetic diagnosis had a much higher risk of mortality (17% estimated ten-year survival) when compared to children with EBV-associated PLPD and a plausible genetic etiology (90% estimated ten-year survival; **Figure 2D**). EBV-LPD may evolve from 1) persistence of EBV-infected lymphocytes as a reflection of immune dysfunction and/or 2) proliferation of EBV-infected lymphocytes that endure despite intact immune function. In this series, the latter was associated with more aggressive disease, including a higher likelihood of HLH, malignancy, and need for HSCT. Early genetic testing may therefore be particularly important for children with EBV-PLPD. Importantly, CAEBV disease is characterized by persistence of EBV without a known immunodeficiency or immune regulation disorder[12]. This distinction underscores the importance of genetic testing in the CAEBV evaluation in order to detect genetic susceptibility to atypical EBV disease/lymphoproliferation and leave CAEBV as a diagnosis of exclusion.

Genetic Diagnoses Yield Treatment Opportunities

Early detection of genetic diagnoses in PLPD informs mechanisms of pathogenesis, facilitates assessments of clinical risks, and identifies potential therapeutic targets. In this PLPD cohort, genetic diagnoses offered improved therapeutic opportunities. Empirically, subjects received treatment with corticosteroids, biologic

therapies, chemotherapy, and/or HSCT upon diagnosis. Results from genetic testing directly led to active changes in the management plan for 12 of the 51 (24%) patients. Unfortunately, 3 subjects died before the potential molecular diagnosis was identified. Specific therapeutic strategies associated with genetic findings are outlined in **Table S6**. Two children (one with activated PI-3-kinase delta syndrome type 1 and one with *CTLA4* haploinsufficiency) received HSCT prior to molecular diagnosis based on clinical features. Overall, our data are consistent with results from a study in which 40% of PIDD patients studied by WES were diagnosed with a genetic cause for disease, leading to changes in the diagnosis and therapeutic management for approximately 25% of patients.

WES also facilitated detection of potential disease-modifying genetic variants. For instance, in addition to a variant of uncertain significance in *CASP1*, siblings LPD010 and LPD023 both carried biallelic variants in *TP53I13* that were computationally predicted to be damaging (**Table S1**). Although this gene is not currently associated with human disease, its gene product is known to have tumor suppressive properties[48]. As a result, we cannot exclude disease contribution from these variants. In a second example, LPD035 was found to have *de novo* and paternally inherited variants in *CDC42* and *NLRP12*, respectively. For this child, anakinra resulted in resolution of fevers, rash, and arthritis but did not alleviate the lymphoproliferative disease, unlike the experience reported by others[27]. This observation is not surprising, since anakinra does not correct the cytoskeletal and cytotoxic abnormalities caused by defects at p.R186 of CDC42[26]. Some of

the improvement observed with anakinra therapy may have occurred due to mitigation of the effect of the *NLRP12* variant. These examples highlight the potential for characterization of molecular defects by WES to inform personalized therapy that may be more effective and safer than empiric immune suppression strategies or HSCT.

Hematopoietic Stem Cell Transplantation in PLPD

The children who underwent HSCT had the lowest ten-year survival (38%) compared to subjects who were given less intense therapies (**Figure S3**), likely reflecting severity of their disease as well as risks of HSCT in patients with uncontrolled lymphoproliferation. Of the 8 children who underwent HSCT, 3 who lacked a genetic explanation proceeded to HSCT due to failure of conventional intervention with empiric steroids, biologics, or cytotoxic chemotherapy. For subjects who survived transplant, 2 of the 3 survivors had genetic diagnoses (*ZBTB24* and *CTLA4* deficiencies). Genetic testing can therefore help to guide the need for this intervention.

Conclusions

Although lymphadenopathy remains a common presentation in children, prolonged and severe symptoms defined by our PLPD criteria characterized a cohort at high risk for mortality for whom no precise diagnostic or therapeutic approach had been established. An unbiased genetic testing approach to delineate the molecular etiologies within our PLPD cohort strongly supports the use of ge-

netic testing to identify potentially actionable disease-causing molecular defects (**Figure 4**)[8]. In particular, significant findings from this study show that genetic testing identified a molecular etiology in 100% of patients with PLPD under one year of age. Further, presence of a genetic error of immunity was associated with improved survival in patients, particularly subjects with EBV associated disease. Lastly, early identification of genetic diagnoses allowed for precision therapy and/or definitive HSCT, potentially avoiding the morbidity and mortality associated with uncontrolled disease and broad immunosuppression. As a result, the findings of the study support early WES and genetic characterization of patients who meet criteria for PLPD both clinically and in prospective cohort studies.

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Author Contributions

LRF, OSE, CEA and IKC conceived of the study, designed experiments, analyzed results and approved the manuscript; LRF, OSE, NG, ECPG analyzed results and drafted the manuscript; NWO, JL, NKEM, MCP, TPV, NSC, NLR, EMM, JSO, JWC, JCAB, SJ, FS, HJC, ASP, HEH, KYK, RHR, DMM, SNJ, RAG, ZHCA, JRL, KLM participated in data review and approved the manuscript.

Conflict of Interest Disclosures

458 The authors have no significant financial interest in or other relationship with the
459 manufacturer of any product or provider of any service mentioned in this article.

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Table 1. Subject Information

Demographics:	
Age at Presentation in Years, median (range)	3.3 (0.08-21)
Sex, *n* (%)	
Male	26 (51.0)
Female	25 (49.0)
Race/Ethnicity, *n* (%)	
Non-Hispanic white	15 (29.4)
Hispanic	25 (49.0)
Non-Hispanic black	2 (3.9)
Non-Hispanic Asian	6 (11.8)
Non-Hispanic other	2 (3.9)
Unknown	1 (2.0)
LPD Characteristics:	
Lymphadenopathy > 3 Months, *n* (%)	
Yes	38 (74.5)
No	13 (25.5)
Lymphocyte Infiltration on Tissue Biopsy	
Yes	12 (23.5)
No	24 (47.0)
Unknown	15 (29.4)
EBV-associated Lymphoproliferation	
Yes	21 (41.2)
No	27 (52.9)
Unknown	3 (5.9)
Associated Clinical Features:	
HLH (5 of 8 criteria), *n* (%)	
Yes	15 (29.4)
No	35 (68.6)
Unknown	1 (2.0)
Autoimmune Disease Diagnosis, *n* (%)	15 (29.4)
Malignancy (following LPD), *n* (%)	4 (7.8)
Splenomegaly, *n* (%)	32 (62.8)
Therapeutic Strategy:	
Maximum Therapeutic Strategy, *n* (%)	
Observation Only	11 (21.6)
Steroid Only	8 (15.7)
Biologics	10 (19.6)
Chemotherapy	11 (21.6)
HSCT	8 (15.7)
Unknown	3 (5.9)
Treated with Rituximab	
Yes	10 (19.6)
No	38 (74.5)
Unknown	3 (5.9)
Outcome:	
Median Follow-up Time in Years, (range)	5.6 (0.10-26.6)
Alive at End of Follow-up, *n* (%)	39 (76.5)

Figure Legends:

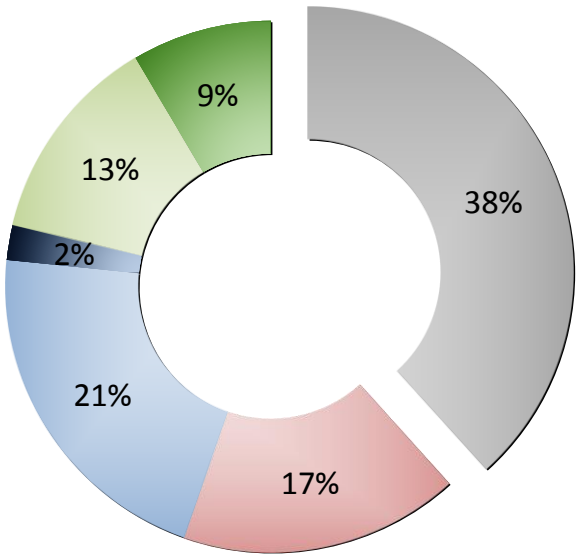
Figure 1. Genetic testing reveals underlying immune defects in children with LPD. Genetic profiles for 47 families who met criteria for PLPD and received whole exome sequencing. The graph displays the distribution of families among the 4 broad genetic categories. The table provides the list of implicated genes (and number of affected families in parentheses, if greater than 1) associated with each defective immune mechanism.

Figure 2. Features of clinical presentation and outcomes. (A) PLPD genetic profile by age at presentation. Subjects were separated into 4 groups by age in years at presentation (*x*-axis). A two-sample test of proportions with a 95% confidence level for each comparison was used to analyze proportional differences in genetic profile by age ($n = 51$). Asterisks indicate a significant ($p < 0.05$) difference from the <1 year old group with the same genetic profile. (B) Ten-year survival estimate from PLPD presentation to date of death or last contact in years ($n = 51$). (C) Ten-year survival estimate from PLPD presentation to date of death or last contact in years by presence of a genetic explanation ($n = 51$). (D) Ten-year survival estimate from PLPD presentation to date of death or last contact in years by EBV-associated disease and genetic explanation ($n = 51$).

Figure 3. Treatment altered by genetic diagnoses. Top part of figure shows the number of subjects eligible for targeted biological therapy alone, hematopoietic stem cell transplantation alone, or either therapy based upon the discovered

genetic diagnosis. Bottom part of figure depicts numbers of patients who were treated according to these strategies before and after genetic testing results became available.

Figure 4. PLPD evaluation and treatment. This schema demonstrates a framework for evaluation and treatment of children with prolonged lymphoproliferation. If symptoms persist or worsen despite standard evaluations and empiric therapies, more extensive laboratory testing characterizing EBV infection status, immune function, and HLH status may be informative. If tissue biopsy demonstrates non-malignant lymphoproliferation, results from this study indicate that genetic evaluations have high likelihood of identifying a genetic cause of disease that may inform optimal therapy ranging from observation to targeted therapy to hematopoietic stem cell transplantation.



- No genetic explanation (n = 18, EBV+ = 7)
- Defective control of lymphocyte activity: pathogenic/likely pathogenic (n = 8, EBV+ = 3)
- Impaired lymphocyte activation, cytoskeletal organization, and apoptosis: pathogenic/likely pathogenic (n = 10, EBV+ = 5)
- Impaired lymphocyte activation, cytoskeletal organization, and apoptosis: VUS (n = 1, EBV+ = 0)
- Dysregulated inflammation: pathogenic/likely pathogenic (n = 6, EBV+ = 2)
- Dysregulated inflammation: VUS (n = 4, EBV+ = 2)

Defective control of lymphocyte activity: pathogenic/likely pathogenic	*BCL6B*, *CTLA4* (x 2), *LRBA*, *PIK3CD* (x 3), *PIK3R1*
Impaired lymphocyte activation, cytoskeletal organization, and apoptosis: pathogenic/likely pathogenic	*CD27* (x 2), *CDC42*, *DOCK4, FAS*, *KRAS, NCKAP1L*, *NRAS*, *RAB27A*, *ZBTB24*
Impaired lymphocyte activation, cytoskeletal organization, and apoptosis: VUS	*IKZF1*
Dysregulated inflammation: pathogenic/likely pathogenic	*CASP1*, *STAT1*, *STAT3* (x 2), *XIAP* (x 2)
Dysregulated inflammation: VUS	*BIRC6*, *CASP1*, *CASP5*, *PLCG2*

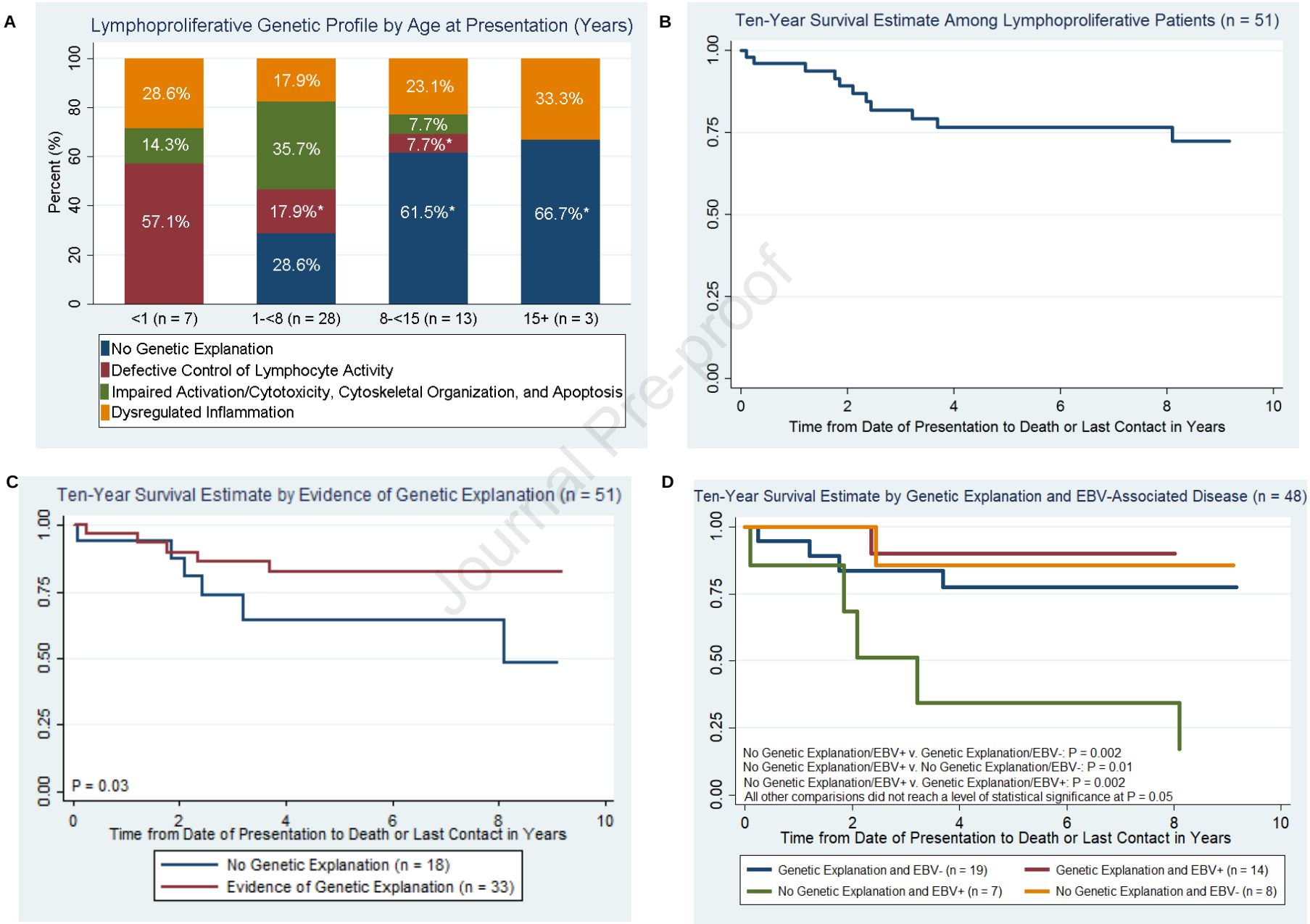
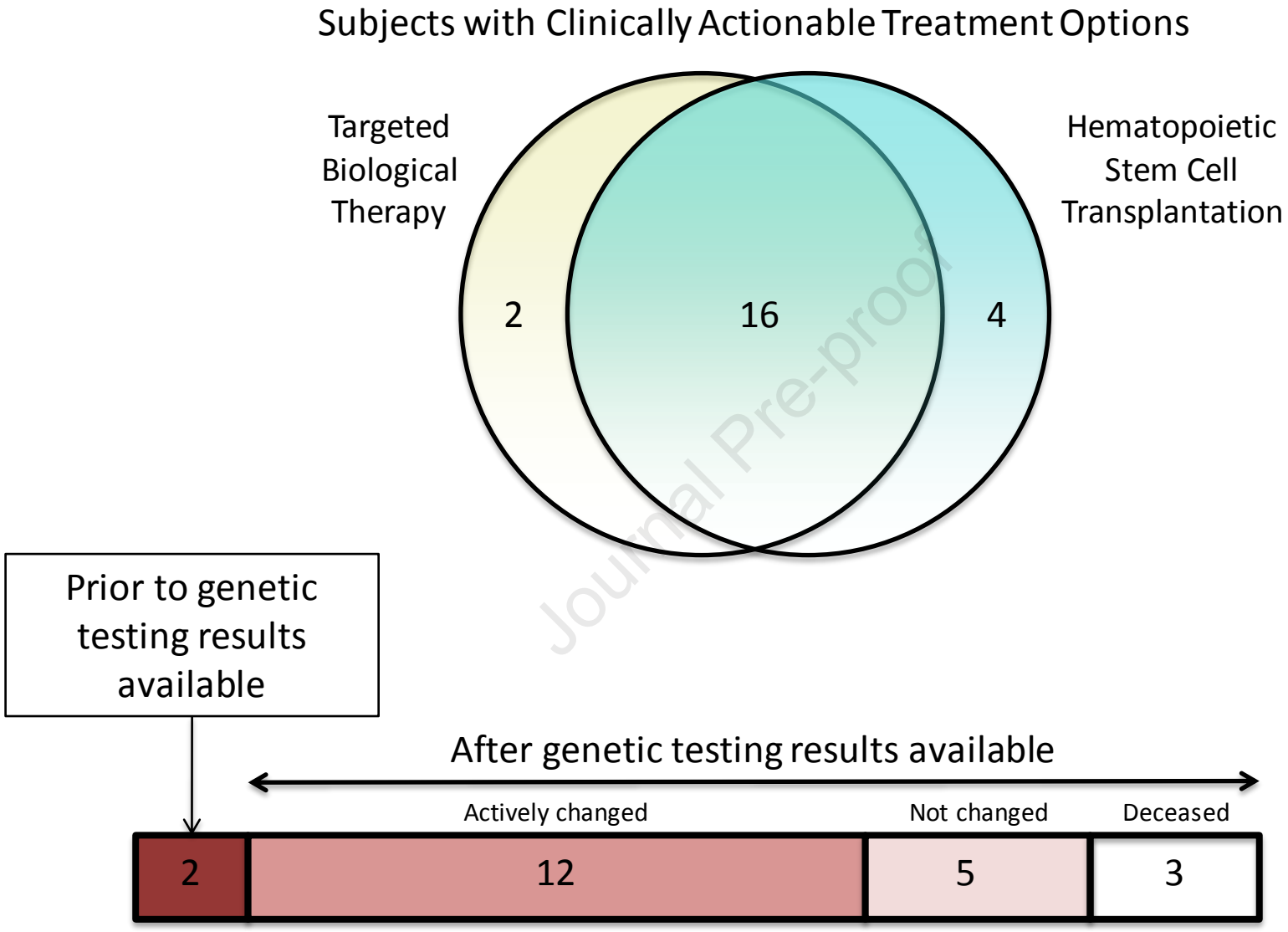


Figure 2



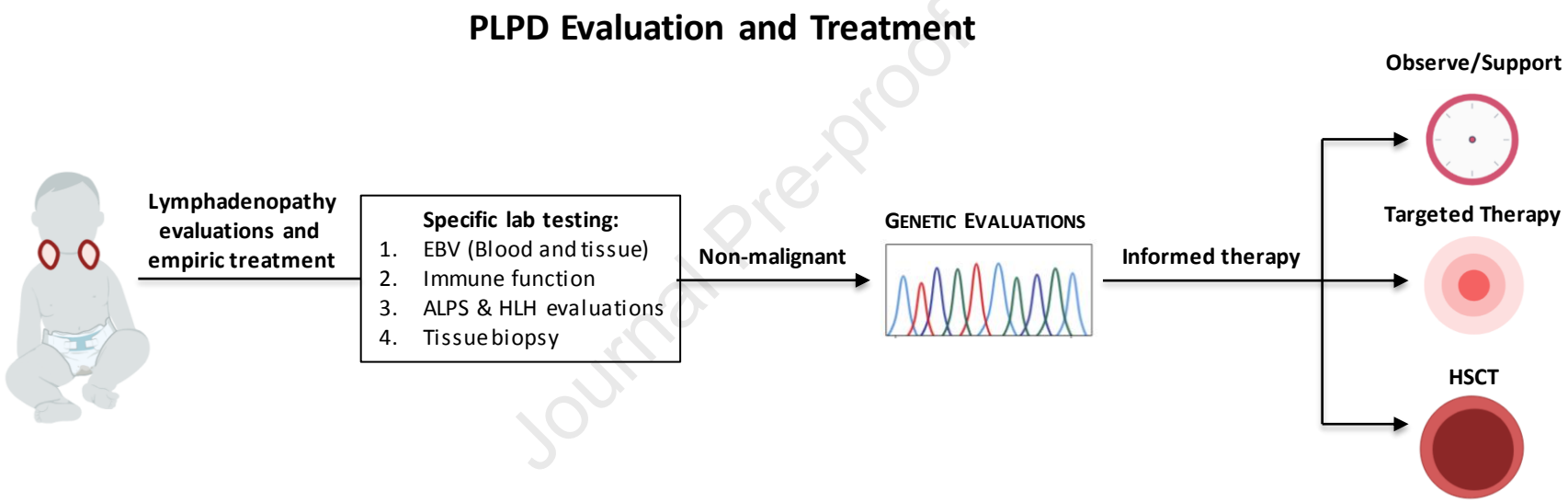


Figure 3