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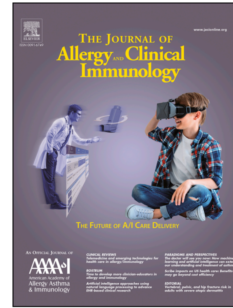

Genetic errors of immunity distinguish pediatric non-malignant lymphoproliferative disorders

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Genetic errors of immunity distinguish pediatric non-malignant lymphoproliferative disorders

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1 **Abstract**

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

6 **Objective:** The primary objective of this study was to ascertain the spectrum of
7 genomic immunologic defects in PLPD. Secondary objectives included character-
8 ization of clinical outcomes and associations between genetic diagnoses and
9 those outcomes.

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

14 **Results:** WES identified likely genetic errors of immunity in 51% to 62% of fami-
15 lies (53% to 65% of affected children). Presence of a genetic etiology was asso-
16 ciated with younger age and hemophagocytic lymphohistiocytosis. Ten-year sur-
17 vival for the cohort was 72.4%, and patients with viable genetic diagnoses had a
18 higher survival rate (82%) compared to children without a genetic explanation
19 (48%, $p = 0.03$). Survival outcomes for individuals with EBV-associated disease
20 and no genetic explanation were particularly worse than outcomes for subjects
21 with EBV-associated disease and a genetic explanation (17% vs. 90%; p =

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22 0.002). Ascertainment of a molecular diagnosis provided targetable treatment op-
23 tions for up to 18 individuals and led to active management changes for 12 pa-
24 tients.

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

27

28 **Clinical Implications**

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

32

33 **Capsule Summary**

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

38

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

41

42 **Abbreviations**

43 ALPS - autoimmune lymphoproliferative syndrome
44 CAEBV - chronic active EBV

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45 CMG - Center for Mendelian Genomics

46 EBV - Epstein-Barr virus

47 EBV-PLPD - EBV-associated PLPD

48 HGSC - Human Genome Sequencing Center

49 HLH - hemophagocytic lymphohistiocytosis

50 HSCT - hematopoietic stem cell transplantation

51 IUIS - International Union of Immunological Societies

52 NK - natural killer

53 PIDD - primary immunodeficiency disease

54 PIRD - primary immune regulatory disorder

55 PLPD - pediatric non-malignant pediatric lymphoproliferative disorders

56 WES - whole exome sequencing

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57 **Introduction**

58 Lymphadenopathy is common during normal childhood and noted on physical
59 examination of approximately half of all children visiting a medical provider for
60 either "well" or "sick" visits.[1] While transient lymphadenopathy in children is rarely
61 dangerous, long-standing lymphoproliferation may reflect underlying immune
62 dysregulation, increase the risk for developing malignant disease or hemophago-
63 cytic lymphohistiocytosis (HLH), and/or drive life-threatening lymphoproliferative
64 disease.[1-3]
65
66 Non-malignant pediatric lymphoproliferative disorders (PLPD) constitute a clini-
67 cally and genetically heterogeneous group of conditions associated with a wide
68 range of clinical consequences. PLPD are characterized by proliferating (and/or
69 persistent) clonal or polyclonal lymphoid cells that may arise as aberrant re-
70 sponses to immune stimuli or represent intrinsic immune dysregulation.[4] Clinical
71 presentations include chronic or recurrent lymphadenopathy, splenomegaly, or
72 symptoms secondary to organ infiltration by abnormal lymphoid cells. In some
73 cases, patients may develop pathologic inflammation consistent with HLH or
74 macrophage activation syndrome. PLPD are also associated with an increased
75 predisposition toward developing hematopoietic malignancies, specifically lym-
76 phoma.[5-7] When a lymph node biopsy is negative for malignancy, the diagnostic
77 and therapeutic paths forward for children with evidence of lymphoproliferation
78 remain poorly defined.
79

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80 Although several inherited diseases of immune dysregulation have been associ-
81 ated with PLPD, the frequency and distribution of primary immunodeficiency dis-
82 eases (PIDD) and primary immune regulatory disorders (PIRD) in children with
83 PLPD are unknown. PIRDs encompass immune mediated disease leading to au-
84 toimmune disease and autoinflammatory conditions[8, 9]. Errors in more than 400
85 genes are now ascribed to PIDD and PIRD[2, 8], and a significant number of these
86 conditions present with clinical features consistent with PLPD.
87
88 PLPD associated with Epstein-Barr virus (EBV) can represent *de novo* infection,
89 reactivation, and/or malignant transformation[7, 10]. PIDD patients who have im-
90 paired natural killer (NK) cell cytotoxic function may have increased susceptibility
91 to primary infection or reactivation of viruses, including EBV[11]. Patients with
92 chronic active EBV (CAEBV), a rare form of EBV disease characterized by per-
93 sistent and/or proliferative EBV-infected lymphocytes during primary or reactivat-
94 ed EBV infection[12], have poor outcomes, especially individuals with EBV specifi-
95 cally detected in NK and T cells[12, 13].
96
97 Optimal management of PLPD patients requires understanding of underlying
98 pathogenic drivers. Given the rare occurrence of PLPD and its overlapping fea-
99 tures with ordinary reactive lymphadenopathy in children, diagnosis is often quite
100 challenging. We therefore sought to determine the utility of whole exome se-
101 quencing (WES) in children with PLPD with a focus on impact on treatment and
102 prognosis.

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103 **Methods**

104 ***Subject Enrollment***

105 Patients and family members at Texas Children's Hospital or collaborating refer-
106 ral centers who met criteria for PLPD between 1994 to 2018 were offered partici-
107 pation in this study. Studies were performed under research protocols approved
108 by the Baylor College of Medicine Institutional Review Board. All procedures in-
109 volving human participants were performed in accordance with institutional and
110 international ethical standards.

111

112 ***Clinical Data and Study Criteria***

113 "PLPD" was defined as persistent lymphadenopathy, lymph organ involvement,
114 or organ lymphocytic infiltration with duration greater than 3 months, with or with-
115 out chronic or significant EBV infection in children and young adults (≤21 years).
116 Chronic or significant EBV infection was defined by recurrent or persistent EBV
117 viremia greater than 3 months, invasive EBV disease, or EBV DNA copy number
118 >100,000 in either whole blood or plasma[13, 14]. Exclusion criteria consisted of his-
119 tory of hematopoietic cell transplantation, solid organ transplantation, established
120 diagnosis of autoimmune lymphoproliferative syndrome (ALPS), or malignancy
121 prior to PLPD. Biopsy details are included in **Supplemental File: Master Data**
122 **Table.** Data regarding co-morbidities and clinical outcomes were extracted from
123 the medical record.

124

125 ***Whole Exome Sequencing and Data Analysis***

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

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149 with additional attention to variants in genes established by the International Un-
150 ion of Immunological Societies (IUIS)[2, 8] to be defective in human immunologic
151 disorders or closely associated with these genes in known protein interactions or
152 immunologic pathways (**Table S1**). Genetic variants were ultimately assigned to
153 the following categories describing potential contributions to immune pathogene-
154 sis: 1) defective control of lymphocyte activity; 2) impaired activation/cytotoxicity,
155 cytoskeletal organization and apoptosis; and 3) dysregulated inflammation.
156

157 ***Statistical Analysis***

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

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164 **Results**

165 ***Characteristics of PLPD Cohort***

166 *Clinical Features*

167 Overall, 51 subjects from 47 families met criteria for PLPD at Texas Children's
168 Hospital and referring centers (**Table 1**). The median age at disease presenta-
169 tion was 3.3 years (range 4 weeks – 21 years) with nearly equal proportions of
170 males (n = 26) and females (n = 25). Almost half (49%) of subjects were Hispan-
171 ic, and 29% were non-Hispanic white. All patients met at least one PLPD criteri-
172 on: 38 patients (74%) had lymphadenopathy for longer than 3 months, 32 pa-
173 tients (63%) had splenomegaly, and 12 patients (23%) had non-malignant lym-
174 phoproliferation on tissue biopsy. Therapeutic strategies ranged from observation
175 to hematopoietic stem cell transplantation (HSCT). Maximum interventions in as-
176 cending order included observation (21.6%), steroids only (15.7%), biologics
177 (19.6%), chemotherapy (21.6%), and HSCT (15.7%).

178

179 *Hemophagocytic lymphohistiocytosis and EBV*

180 Among the 51 subjects, 15 patients (29%) fulfilled at least five of eight HLH-
181 2004[22] diagnostic criteria for HLH: 9 (60%) survived, and 8 (53%) had EBV-
182 associated disease (**Table 1, Table S2**). Among the entire cohort, 21 (41%) had
183 EBV-PLPD and 14 (67%) of these patients survived (**Table 1, Table S3**). Five of
184 8 (63%) patients with both EBV-PLPD and HLH survived, and 9 of 12 (75%) pa-
185 tients with EBV-PLPD without HLH survived.

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187 *Autoimmune and Autoinflammatory Conditions*

188 Fifteen subjects (29%) were diagnosed with autoimmune and/or autoinflammato-
189 ry conditions either prior to or concurrent with their PLPD diagnosis (**Table 1,**
190 **Table S4**), and this subset of patients had an overall survival rate of 73%. Of the
191 22 subjects who had testing for double negative alpha-beta T cells, 11 had ele-
192 vated levels (≥ 1.5% of total lymphocytes). ALPS was considered at some point
193 in the medical record in 40 patients (78%), but upon evaluation none in this co-
194 hort met diagnostic criteria[23, 24] prior to enrollment, and no functional defects in
195 apoptosis were identified. However, ALPS-associated gene defects were subse-
196 quently identified in 2 patients in whom ALPS was not initially suspected or eval-
197 uated. For reference, 14 patients were diagnosed with ALPS at our institution
198 during the study period (and were therefore excluded from this cohort).

199

200 *Malignancy*

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

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209 lymphoproliferative disease, EBV infection, or immune deficiency, survived
210 (25%).
211
212 ***Genetic Findings***
213 *Genetic Errors of Immunity are Prevalent in PLPD*
214 All 51 participants from the 47 families underwent WES. Clinical WES was com-
215 pleted in 19 of the families (19 probands), resulting in genetic diagnoses for only
216 4 children (21%). For the other 15 cases and families who underwent clinical
217 WES which did not yield a diagnosis, 12 consented to research-level analyses of
218 the clinical exome data, resulting in identification of an additional 8 candidate mo-
219 lecular diagnoses. For one of these families, research WES of 2 additional af-
220 fected siblings enabled identification of the defect in *PIK3CD* in all 3 children.
221 Research-based WES analyses were also performed without clinical WES for 28
222 families (30 cases), leading to likely molecular diagnoses in 13 (46%) [14 cases,
223 47%] and further potential genetic explanations in 4 (14%) [5 cases, 17%]. Thus,
224 29 of 47 PLPD families (62%), or 33 of 51 affected children (65%), were found to
225 have likely or plausible disease-associated genetic errors of immunity (**Table**
226 **S1**). Note that "genetic errors" serves as a more appropriate term than "inborn
227 errors" because of the identified likely somatic changes to *KRAS* and *NRAS.* Of
228 these 29 families (33 cases) with viable genetic explanations, 21 (23 cases) had
229 disease candidate variants in 15 IUIS-established PIDD and PIRD genes[2, 3, 8].
230 One family (LPD019 and LPD034) was discovered to have a novel disease can-
231 didate 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

255 genetic explanation for the disease. Of the 28 patients between 1 and 8 years of
256 age, 72% had a potential genetic etiology identified. In contrast, a molecular di-
257 agnosis for PLPD was less likely to be identified in the 16 patients who devel-
258 oped symptoms after 8 years of age (38%).
259
260 The proportion of patients with possible genetic explanations did not differ signifi-
261 cantly between EBV-PLPD and PLPD without EBV. Of the 21 patients with EBV-
262 PLPD, 67% had potential genetic explanations, and of the 27 patients with PLPD
263 without EBV, 70% had implicated genetic findings (p = 0.91). Likewise, among
264 the three immune-mediated genetic categories, the proportion of EBV-affected
265 individuals was evenly distributed (**Figure 1**).
266
267 Genetic findings were more common in patients with HLH compared to patients
268 who eventually developed malignancy, although the proportional differences did
269 not reach a level of statistical significance (p = 0.08). Among the 15 patients who
270 met HLH diagnostic criteria[13, 22], a probable genetic explanation was present in
271 11 (73%), 9 of whom were under the age of 8 (**Table S2**). Fewer patients who
272 developed malignancy subsequent to their PLPD diagnosis (25%) had a genetic
273 disorder (**Table S5**).
274
275 *Lack of Genetic Diagnosis is Associated with Increased Risk for Mortality*
276 Estimated ten-year survival for the entire cohort was 72.4% with a median follow-
277 up of 5.6 years (range 0.10 - 26.6 years, **Figure 2B**). Analyzing the cohort as a

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278 whole (**Figure 2C, Figure S2**), patients without an identified possible genetic eti-
279 ology had significantly lower ten-year survival compared to patients with a poten-
280 tial genetic explanation (48% versus 82%, respectively, $p = 0.03$). The ten-year
281 survival estimate for children with EBV-PLPD trended lower compared to children
282 without EBV (56% vs 80%; $p = 0.13$). Children with EBV-PLPD frequently had
283 complicated courses: 5 had HLH, 4 developed malignancy, and 1 developed both
284 malignancy and HLH. Presence of EBV-PLPD did not predict an underlying ge-
285 netic defect. Most notably, however, subjects with EBV-PLPD without a viable
286 genetic explanation had significantly lower estimated survival than children with a
287 suggested genetic explanation (17% vs. 90%, $p = 0.002$; **Figure 2D**). In fact, the
288 group of patients who had EBV-PLPD without a genetic explanation was the cat-
289 egory associated with the highest risk of death.
290
291 *Genetic Testing Impacts Therapeutic Decisions*
292 Identification of an underlying genetic diagnosis in PLPD patients informs thera-
293 peutic opportunities (**Figure 4, Table S6**). Currently, targeted therapies are
294 available or show promise for treatment of at least 11 of the genetic conditions
295 diagnosed in this cohort (potentially benefitting up to 18 patients from 16 fami-
296 lies)[28]. Furthermore, successful outcomes have been reported after HSCT in 10
297 of the 15 IUIS-recognized genetic errors of immunity reported here (which could
298 treat up to 20 patients from 18 families). Prior to the availability of genetic testing
299 results, only two patients had received empiric treatment that would have been
300 supported by their ultimate genetic diagnoses. After genetic testing results were

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

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311 **Discussion**

312 *Clinical and Genomic Landscape of PLPD*

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

321

322 In order to improve knowledge of underlying immune pathogenesis mechanisms
323 in PLPD to better inform treatment, we performed WES of 51 subjects from the
324 47 families in this cohort. This unbiased approach led to a genetic diagnosis in
325 51% to 62% of families [53% to 65% of affected children] (**Figure 1**), encapsulat-
326 ing a heterogenous collection of genetic errors of immunity. As a comparison,
327 Stray-Pedersen et al reported a 40% overall genetic diagnostic rate, including
328 potentially novel diseases, in patients with PIDD.[18] Findings from this study sup-
329 port the clinical utility of comprehensive genetic analysis in PLPD, with high like-
330 lihood of identifying genetic alterations that inform therapeutic opportunities and
331 clinical risk.

332

333 *PLPD Risk Stratification*

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334 Overall survival was 72% with a trend towards worse outcomes associated with
335 EBV infection, HLH, and subsequent malignancy. Earlier age at presentation with
336 LPD positively correlated with likelihood of identifying a potential genetic diagno-
337 sis, especially in children with impaired lymphocyte activation/cytotoxicity, cyto-
338 skeletal organization, and apoptosis (**Table S7**). In fact, a molecular explanation
339 was found in all 7 patients who presented at less than 1 year of age. These data
340 particularly support the clinical utility of WES for infants and younger children with
341 PLPD. At older ages, acquired factors, such as autoimmune disease and infec-
342 tion, may also contribute to development of PLPD. Even so, for 9 patients above
343 12 years of age, 3 had a plausible underlying genetic explanation, suggesting
344 that genetic testing can play a critical role in diagnosis and management of PLPD
345 in adolescents and young adults as well.
346
347 *Increased Mortality in Patients with EBV-PLPD and No Genetic Explanation*
348 EBV is the most common pathogen associated with non-malignant LPD[32]. In this
349 cohort, patients with EBV-PLPD had pathogenic or likely pathogenic variants in
350 several genes associated with atypical EBV disease: *CTLA4, LRBA, PIK3CD,*
351 *CD27, RAB27A, ZBTB24,* and *STAT1*[33]. Additionally, somatic *PLCG2* mutations
352 have correlated with EBV-positive Burkitt lymphoma[34]. Potentially disease-
353 associated variants in *CASP1* and *CASP5* were also discovered in EBV-PLPD
354 patients [24, 35-46]. CASP1 has provocatively been implicated in IRF8-dependent
355 EBV lytic reactivation[47]. EBV status alone, however, did not impact the likelihood
356 of having a potential underlying genetic explanation for LPD (67% of EBV-

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357 associated LPD vs. 70% of non-EBV-associated LPD). Furthermore, susceptibil-
358 ity to EBV infection was not significantly skewed toward any of the three immuno-
359 logic mechanism categories (**Figure 1**). However, children with EBV-associated
360 PLPD without an identifiable genetic diagnosis had a much higher risk of mortali-
361 ty (17% estimated ten-year survival) when compared to children with EBV-
362 associated PLPD and a plausible genetic etiology (90% estimated ten-year sur-
363 vival; **Figure 2D**). EBV-LPD may evolve from 1) persistence of EBV-infected
364 lymphocytes as a reflection of immune dysfunction and/or 2) proliferation of EBV-
365 infected lymphocytes that endure despite intact immune function. In this series,
366 the latter was associated with more aggressive disease, including a higher likeli-
367 hood of HLH, malignancy, and need for HSCT. Early genetic testing may there-
368 fore be particularly important for children with EBV-PLPD. Importantly, CAEBV
369 disease is characterized by persistence of EBV without a known immunodefi-
370 ciency or immune regulation disorder[12]. This distinction underscores the im-
371 portance of genetic testing in the CAEBV evaluation in order to detect genetic
372 susceptibility to atypical EBV disease/lymphoproliferation and leave CAEBV as a
373 diagnosis of exclusion.

374

375 *Genetic Diagnoses Yield Treatment Opportunities*

376 Early detection of genetic diagnoses in PLPD informs mechanisms of pathogen-
377 esis, facilitates assessments of clinical risks, and identifies potential therapeutic
378 targets. In this PLPD cohort, genetic diagnoses offered improved therapeutic op-
379 portunities. Empirically, subjects received treatment with corticosteroids, biologic

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380 therapies, chemotherapy, and/or HSCT upon diagnosis. Results from genetic
381 testing directly led to active changes in the management plan for 12 of the 51
382 (24%) patients. Unfortunately, 3 subjects died before the potential molecular di-
383 agnosis was identified. Specific therapeutic strategies associated with genetic
384 findings are outlined in **Table S6**. Two children (one with activated PI-3-kinase
385 delta syndrome type 1 and one with *CTLA4* haploinsufficiency) received HSCT
386 prior to molecular diagnosis based on clinical features. Overall, our data are con-
387 sistent with results from a study in which 40% of PIDD patients studied by WES
388 were diagnosed with a genetic cause for disease, leading to changes in the diag-
389 nosis and therapeutic management for approximately 25% of patients.
390
391 WES also facilitated detection of potential disease-modifying genetic variants.
392 For instance, in addition to a variant of uncertain significance in *CASP1*, siblings
393 LPD010 and LPD023 both carried biallelic variants in *TP53I13* that were compu-
394 tationally predicted to be damaging (**Table S1**). Although this gene is not current-
395 ly associated with human disease, its gene product is known to have tumor sup-
396 pressive properties[48]. As a result, we cannot exclude disease contribution from
397 these variants. In a second example, LPD035 was found to have *de novo* and
398 paternally inherited variants in *CDC42* and *NLRP12*, respectively. For this child,
399 anakinra resulted in resolution of fevers, rash, and arthritis but did not alleviate
400 the lymphoproliferative disease, unlike the experience reported by others[27]. This
401 observation is not surprising, since anakinra does not correct the cytoskeletal
402 and cytotoxic abnormalities caused by defects at p.R186 of CDC42[26]. Some of

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403 the improvement observed with anakinra therapy may have occurred due to miti-
404 gation of the effect of the *NLRP12* variant. These examples highlight the potential
405 for characterization of molecular defects by WES to inform personalized therapy
406 that may be more effective and safer than empiric immune suppression strate-
407 gies or HSCT.
408
409 *Hematopoietic Stem Cell Transplantation in PLPD*
410 The children who underwent HSCT had the lowest ten-year survival (38%) com-
411 pared to subjects who were given less intense therapies (**Figure S3**), likely re-
412 flecting severity of their disease as well as risks of HSCT in patients with uncon-
413 trolled lymphoproliferation. Of the 8 children who underwent HSCT, 3 who lacked
414 a genetic explanation proceeded to HSCT due to failure of conventional interven-
415 tion with empiric steroids, biologics, or cytotoxic chemotherapy. For subjects who
416 survived transplant, 2 of the 3 survivors had genetic diagnoses (*ZBTB24* and
417 *CTLA4* deficiencies). Genetic testing can therefore help to guide the need for
418 this intervention.
419
420 *Conclusions*
421 Although lymphadenopathy remains a common presentation in children, pro-
422 longed and severe symptoms defined by our PLPD criteria characterized a co-
423 hort at high risk for mortality for whom no precise diagnostic or therapeutic ap-
424 proach had been established. An unbiased genetic testing approach to delineate
425 the molecular etiologies within our PLPD cohort strongly supports the use of ge-

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

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449

450 **Author Contributions**

451 LRF, OSE, CEA and IKC conceived of the study, designed experiments, ana-
452 lyzed results and approved the manuscript; LRF, OSE, NG, ECPG analyzed re-
453 sults and drafted the manuscript; NWO, JL, NKEM, MCP, TPV, NSC, NLR, EMM,
454 JSO, JWC, JCAB, SJ, FS, HJC, ASP, HEH, KYK, RHR, DMM, SNJ, RAG,
455 ZHCA, JRL, KLM participated in data review and approved the manuscript.
456

457 **Conflict of Interest Disclosures**

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458 The authors have no significant financial interest in or other relationship with the
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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)

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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.

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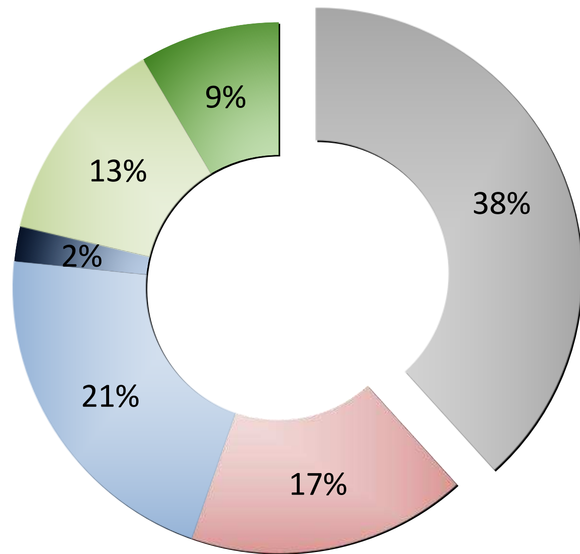

No genetic explanation (n = 18, EBV+ = 7)

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*

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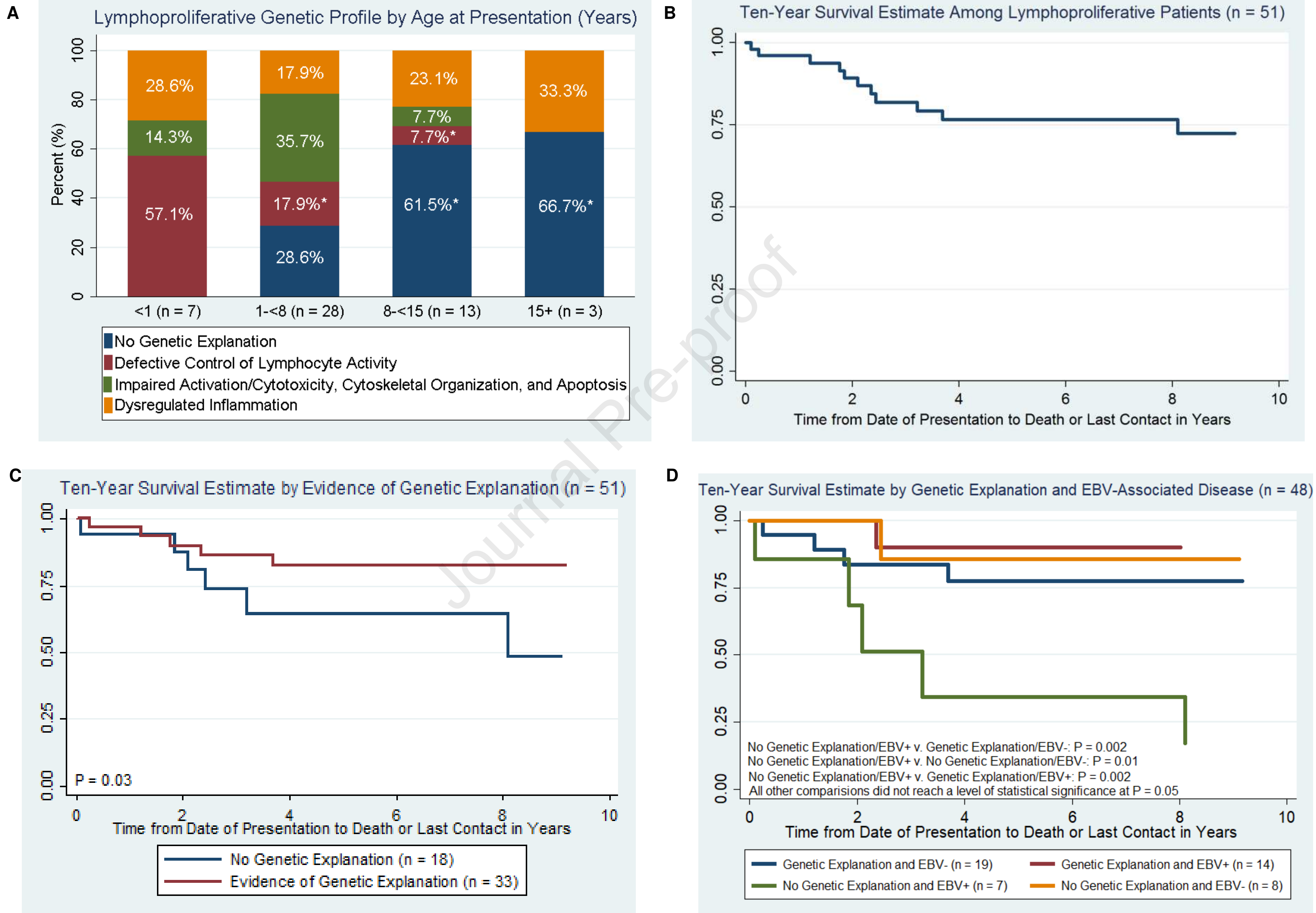

Figure 2

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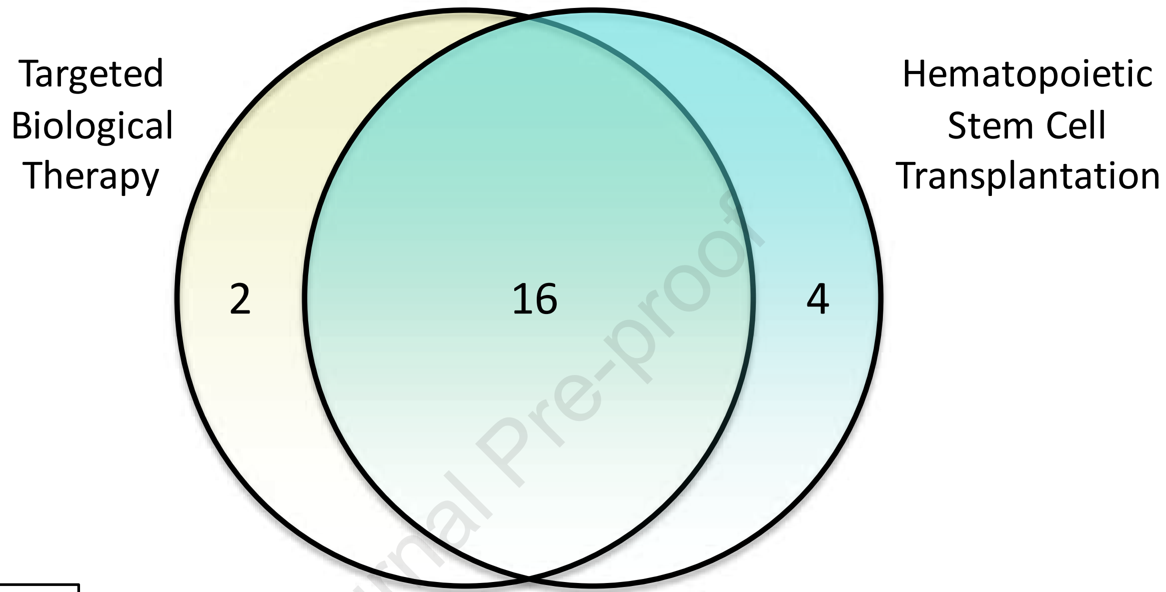

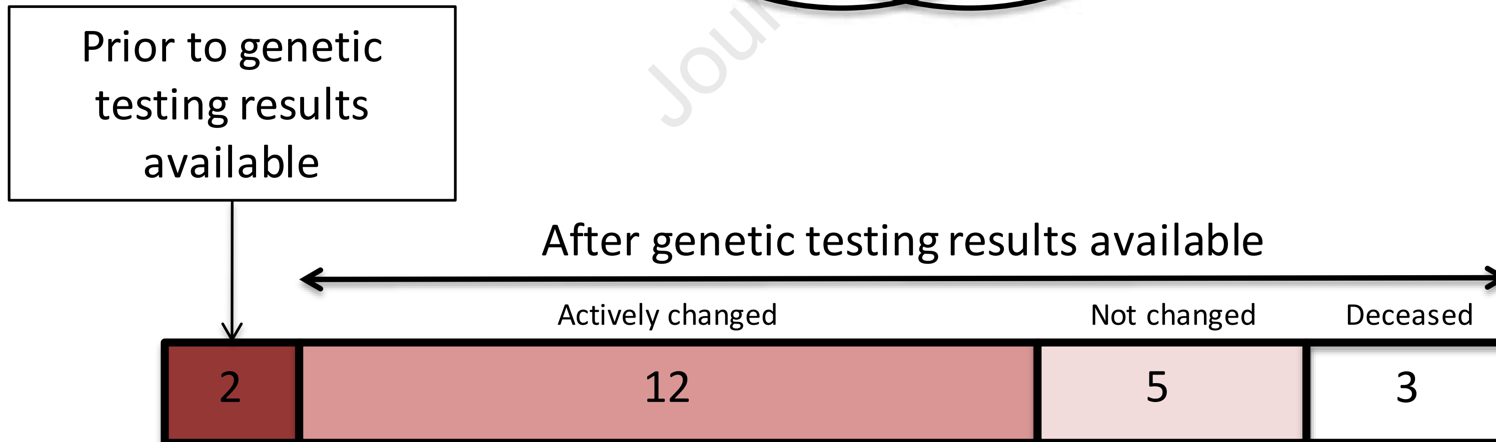

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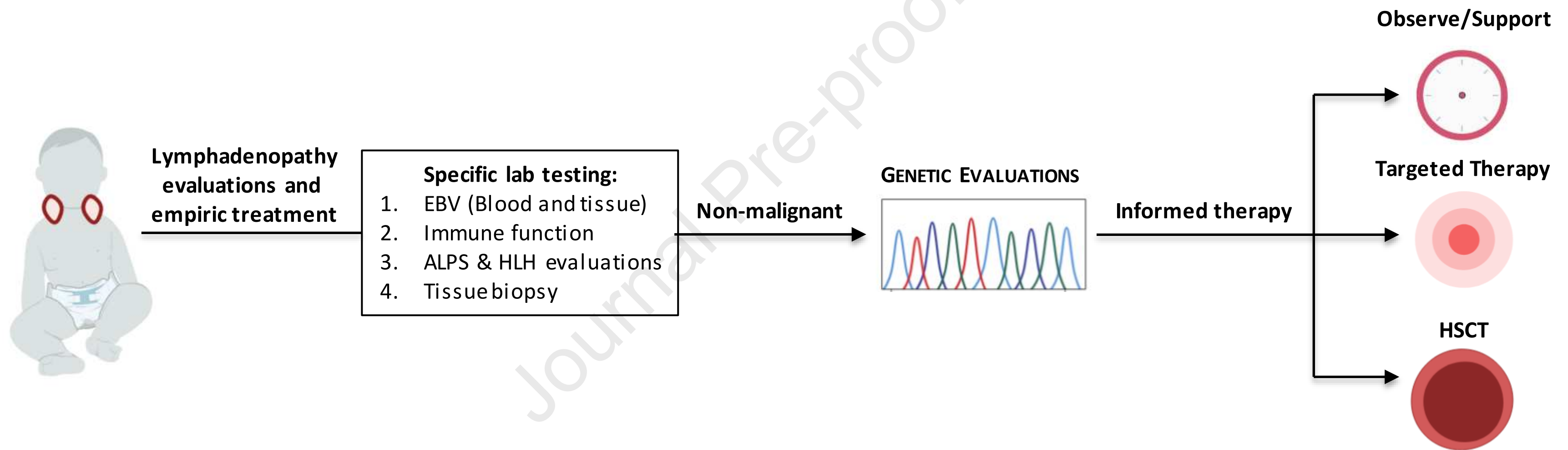

Journal Pre-proof

Figure 3