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## Next-Generation Sequencing in Diffuse Large B-Cell Lymphoma Highlights Molecular Divergence and Therapeutic Opportunities: a LYSA Study

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### ► To cite this version:

Sydney Dubois, Pierre-Julien Vially, Sylvain Mareschal, Elodie Bohers, Philippe Bertrand, et al.. Next-Generation Sequencing in Diffuse Large B-Cell Lymphoma Highlights Molecular Divergence and Therapeutic Opportunities: a LYSA Study. *Clinical Cancer Research*, 2016, 22 (12), pp.2919–2928. 10.1158/1078-0432.CCR-15-2305 . hal-01343064

**HAL Id: hal-01343064**

**<https://univ-rennes.hal.science/hal-01343064>**

Submitted on 15 Dec 2016

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1 **Next Generation Sequencing in Diffuse Large B Cell Lymphoma Highlights Molecular**  
2 **Divergence and Therapeutic Opportunities: a LYSA Study**

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31  
32 **Running Title:** Targeted NGS details DLBCL divergence and actionable targets

33  
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40 **Keywords:** Diffuse Large B Cell Lymphoma, Primary Mediastinal B-cell Lymphoma, Next  
41 Generation Sequencing

42  
43 **Declaration of Interests:** The authors disclose no potential conflicts of interest.

44 **Total word count:** 3866, Abstract word count: 247, Number of figures and tables: 6

49 **Translational Relevance**

50

51 This is the first Next Generation Sequencing (NGS) study of such a large, prospective cohort  
52 using a targeted gene panel, the Lymphopanel, focusing on genes identified as important for  
53 lymphomagenesis or whose potential has been pinpointed in Whole Exome Sequencing  
54 studies. Particular attention has been paid to the inclusion of actionable targets in the  
55 Lymphopanel, highlighting potential candidate patients for novel personalized therapies. This  
56 study further details subtype-enriched gene and pathway mutations in order to optimize  
57 DLBCL treatment, and highlights several novel, frequent, and actionable mutations, notably  
58 in PMBL. In addition, Lymphopanel NGS has enabled the detection of novel clinical and  
59 prognostic correlations, potentially impacting treatment decisions. As benchtop sequencers  
60 become increasingly available in academic research and routine clinical settings, our study  
61 demonstrates the feasibility and impact of NGS with a consensus gene panel in DLBCL  
62 patient management, contributing essential information to today's precision therapy era  
63 treatment decisions.

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84 **Abstract**

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86 **Purpose:** Next Generation Sequencing (NGS) has detailed the genomic characterization of  
87 Diffuse Large B Cell Lymphoma (DLBCL) by identifying recurrent somatic mutations. We  
88 set out to design a clinically feasible NGS panel focusing on genes whose mutations hold  
89 potential therapeutic impact. Furthermore, for the first time, we evaluated the prognostic value  
90 of these mutations in prospective clinical trials.

91 **Experimental Design:** A Lymphopanel was designed to identify mutations in 34 genes,  
92 selected according to literature data and a whole exome sequencing study of  
93 relapsed/refractory DLBCL patients. The tumor DNA of 215 patients with CD20+ *de novo*  
94 DLBCL in the prospective, multicenter and randomized LNH-03B LYSA clinical trials was  
95 sequenced to deep, uniform coverage with the Lymphopanel. Cell of origin molecular  
96 classification was obtained through gene expression profiling with HGU133+2.0 Affymetrix  
97 GeneChip arrays.

98 **Results:** The Lymphopanel was informative for 96% of patients. A clear depiction of DLBCL  
99 subtype molecular heterogeneity was uncovered with the Lymphopanel, confirming that  
100 Activated B Cell-like (ABC), Germinal Center B-cell like (GCB) and Primary Mediastinal B-  
101 cell Lymphoma (PMBL) are frequently affected by mutations in NFkB, epigenetic, and JAK-  
102 STAT pathways respectively. Novel truncating immunity pathway, *ITPKB*, *MFHAS1* and  
103 *XPO1* mutations were identified as highly enriched in PMBL. Notably, *TNFAIP3* and *GNAI3*  
104 mutations in ABC patients treated with R-CHOP were associated with significantly less  
105 favorable prognoses.

106 **Conclusions:** This study demonstrates the contribution of NGS with a consensus gene panel  
107 to personalized therapy in DLBCL, highlighting subtypes' molecular heterogeneity and  
108 identifying somatic mutations with therapeutic and prognostic impact.

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119 **Introduction**

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121 Diffuse large B-cell lymphoma (DLBCL) is the most common form of adult lymphoma  
122 worldwide, accounting for 30-40% of newly diagnosed Non-Hodgkin Lymphoma (NHL)(1).  
123 Gene expression profiling (GEP) has made strides in deciphering the molecular heterogeneity  
124 of DLBCL, enabling the entity's subdivision into three main molecular subtypes: Germinal  
125 Center B-cell like (GCB), Activated B-Cell like (ABC), and Primary Mediastinal B-cell  
126 Lymphoma (PMBL)(2,3). Among other genetic aberrations, the GCB subtype is characterized  
127 by t(14;18)(q32;q21) translocations(4) and loss of *PTEN*(5), while the ABC subtype is  
128 characterized by t(3;14)(q27;q32) translocations, deletion of the *INK4A-ARF* locus(6) and  
129 *BCL2* amplification(7). PMBL displays strong molecular similarities to classical Hodgkin  
130 Lymphoma (cHL), exhibiting frequent amplifications of *JAK2* and deletions of *SOCS1*(8,9).  
131 Arising from B cells at distinct stages of differentiation and maturation, these subtypes are  
132 also diverse in clinical presentation, response to immunochemotherapy and outcome, with the  
133 ABC subtype having the most unfavorable prognosis(2,7). As targeted therapies become  
134 increasingly widespread, it is essential to thoroughly characterize each molecular subtype, in  
135 order to ensure optimal care for each patient. Unfortunately, the cell of origin (COO)  
136 classification of DLBCL still has relatively little influence on clinical practice, as GEP is  
137 poorly transposable to routine diagnosis and surrogate immunohistochemical algorithms  
138 remain unreliable(10), although recent techniques offer better bench to bedside  
139 translation(11,12).

140 Next generation sequencing (NGS) technologies, enabling high-throughput DNA sequencing,  
141 have emerged over the past decade and have provided new insights into the genomic  
142 characterization of DLBCL by identifying recurrent single nucleotide variants (SNVs), which  
143 can be enriched in a particular subtype(13–22). Although DLBCL is defined by widespread  
144 genetic heterogeneity, several of the pinpointed recurrent SNVs warrant special interest as  
145 they occur in actionable targets and/or correlate with antitumoral response. Equally of note,  
146 despite their probable physiopathological relevance, neither the prognostic value of these  
147 mutations nor their potential to predict resistance to conventional B-cell lymphoma  
148 immunochemotherapy treatment has been properly evaluated in prospective clinical trials.  
149 Furthermore, mutation feature analyses have identified activation-induced cytidine deaminase  
150 (AID)-driven somatic hypermutation (SHM) as a mechanism mediating the development of

151 some of these recurrent SNVs, rendering the task of distinguishing driver from passenger  
152 mutations even more challenging(23).

153 To more feasibly identify molecular subtypes and reach the goal of precision therapy in  
154 DLBCL, a limited and clinically relevant panel of target genes must be designed, with efforts  
155 made to thoroughly describe the SNVs they harbor. Furthermore, compact sequencers readily  
156 available in academic laboratories must be used in order to generalize this practice and apply  
157 it to routine clinical disease management. With this idea in mind, we developed a  
158 Lymphopanel NGS assay, designed to identify mutations in 34 genes important for  
159 lymphomagenesis based on a literature review of WES studies in DLBCL(13,14,20,24) as  
160 well as a WES study of relapsed/refractory DLBCL cases(25). In the current study, we  
161 sequenced 215 patients with *de novo* DLBCL enrolled in the prospective, multicenter and  
162 randomized LYSA LNH-03B trials, using the Lymphopanel. Our intent was to determine  
163 whether the Lymphopanel was informative enough to identify targetable SNVs and/or  
164 pathway mutations that might alter treatment decisions, highlight subtype-specific mutational  
165 profiles, distinguish genes impacted by AID, and potentially foretell clinical outcome.

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## 168 **Materials and Methods**

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### 170 **Patients**

171 215 adult patients with *de novo* CD20+ DLBCL enrolled in the prospective, multicenter and  
172 randomized LNH-03B LYSA trials with available frozen tumor samples, centralized  
173 histopathological review and adequate DNA/RNA quality were selected. The LNH03-B  
174 LYSA trials were initiated in 2003 and included 1704 patients overall in six distinct clinical  
175 trials (Supplementary Methods). COO molecular classification was obtained with  
176 HGU133+2.0 Affymetrix GeneChip arrays (Affymetrix), grouping patients into ABC, GCB,  
177 PMBL and “other”, also referred to as unclassified (detailed in Supplementary Methods).  
178 Clinical features of the patients are indicated in Table S1. The study was performed with  
179 approval of an institutional review board and written informed consent was obtained from all  
180 participants at the time of enrollment.

181

### 182 **NGS experiments and data analysis**

183 The Lymphopanel was designed to identify mutations in 34 genes important for  
184 lymphomagenesis, based on literature data (Table S2)(24) and WES of relapsed/refractory

185 DLBCL sequencing(25). The design covers 87 703 bases using 872 amplicons. Genes were  
186 grouped into 8 specific pathways: Immunity (*CIITA*, *B2M*, *TNFRSF14* and *CD58*), NOTCH  
187 (*NOTCH1* and *NOTCH2*), Apoptosis/Cell cycle (*MFHAS1*, *XPO1*, *MYC*, *CDKN2A/B*,  
188 *FOXO1*, *TP53*, *GNA13* and *BCL2*), NFkB (*TNFAIP3*, *MYD88*, *PIM1*, *CARD11*, *IRF4* and  
189 *PRDM1*), Epigenetic Regulation (*EZH2*, *KMT2D*, *EP300*, *MEF2B* and *CREBBP*), MAP  
190 Kinase (*BRAF*), JAK-STAT (*SOCS1* and *STAT6*), and BCR (*CD79A/B*, *ITPKB*, *TCF3* and  
191 *ID3*) (Table S3).

192 Ion Torrent Personal Genome Machine (PGM) Sequencing and PGM data analysis was  
193 performed as previously described(26) and detailed in Supplementary Methods. Variant  
194 analysis was performed using an in-house generated bioinformatics pipeline, detailed in  
195 Supplementary Methods and Figures S1 and S2. This pipeline ensured excellent filtering of  
196 artefacts and polymorphisms, as proven by an independent study of seven non-tumoral  
197 samples from DLBCL patients with a mean error rate of non-filtered variants of only 0.7%  
198 (Table S4).

199

## 200 **Statistical methods**

201 All statistical analyses were performed using R software version 3.1.2(27). Progression-Free  
202 survival (PFS) was evaluated from the date of enrolment to the date of disease progression,  
203 relapse, re-treatment or death from any cause. Overall survival (OS) was evaluated from the  
204 date of enrolment to the date of death from any cause. Multivariate Cox and log-rank tests  
205 (“survival” R package version 2.37.7) were used to assess differences in OS and PFS rates  
206 calculated by Kaplan-Meier estimates. Ward hierarchical unsupervised clustering (“LPS” R  
207 package version 1.0.10) was performed using Jaccard distance. Statistical differences between  
208 all other parameters were determined using  $\chi^2$ , Mann-Whitney or Fisher’s exact tests when  
209 appropriate. P values or False Discovery Rates (FDR) < 0.05 were considered statistically  
210 significant.

211

## 212 **Results and Discussion**

213

### 214 **Lymphopanel Variant Features**

215 Lymphopanel NGS was performed on 215 DLBCL patients (81 ABC, 83 GCB, 33 “other”  
216 and 18 PMBL). The median overall sequencing depth was 225x [110x-331x]. An initial  
217 13 965 variants were filtered for quality, SNPs and functional relevance, leading to 1064  
218 (7.6%) variants ultimately validated (Table S5 and Figure S1). The Lymphopanel

219 informativity was of 96%, with 206 patients (78 GCB, 80 ABC, 30 “other” and 18 PMBL)  
220 presenting at least one variant. All genes in the Lymphopanel were informative for at least  
221 one patient, with the number of variants per gene ranging from one to 124. Gene mutation  
222 frequencies in the total cohort and by subtype are presented in Figure 1.

223 We subdivided the genes targeted by the Lymphopanel into eight specific pathways (Figure 2,  
224 Table S3). As expected, we confirmed that the ABC subtype is dominated by NFkB pathway  
225 mutations (45% of total variants), followed by epigenetic regulation pathway mutations  
226 (20.2% of total variants) (Figure 2A), whereas the GCB subtype is mostly characterized by  
227 mutations in the epigenetic regulation pathway (32.3% of total variants) and the apoptosis/cell  
228 cycle pathway (26.3% of total variants) (Figure 2B). Mutations in the JAK-STAT, immunity  
229 and apoptosis/cell cycle pathways were prevalent in PMBL (29.1%, 20.9% and 20.9% of total  
230 variants respectively) (Figure 2C). Interestingly, PMBL presented a significantly higher  
231 number of variants per sample than the other subtypes combined ( $p=2.76 \times 10^{-5}$ , Figure S3).  
232 The “other” subtype showed a fairly hybrid spectrum of pathway mutations (Figure 2D),  
233 perhaps suggesting the involvement of the three main subtypes, rather than the existence of a  
234 distinct entity.

235 We sought to assess the impact of AID involvement in the Lymphopanel genes (detailed in  
236 Supplementary Methods and Figure S4). Genes with an AID mutation frequency superior to  
237 the average of that of the Lymphopanel are presented in Table S6. Previously identified SHM  
238 targets in DLBCL among these genes include *MYC*, *PIM1*, *IRF4*, *BCL2*, *SOCS1* and  
239 *CIITA*(23). Potentially novel SHM targets include *MFHAS1*, *PRDMI*, *GNA13*, *MYD88*,  
240 *ITPKB* and *NOTCH2*. There was no significant difference in AID involvement between  
241 DLBCL subtypes.

242

### 243 **Lymphopanel NGS identifies mutations with potential treatment impact**

244 The Lymphopanel included genes whose mutations could serve to stratify patients according  
245 to treatment options. This includes actionable mutations, currently targeted by precision  
246 therapies, highlighted in Figure 1, as well as mutations whose presence might call into  
247 question the use of certain targeted therapy options.

248 Recent results have shown that doubly mutated *MYD88/CD79B* patients are significantly  
249 more responsive to single therapy Ibrutinib, a BTK inhibitor, highlighting the importance of  
250 targeted NGS for DLBCL patients in the clinical setting(28). Furthermore, the presence of  
251 *CARD11* and *TNFAIP3* mutations has been shown to lead to decreased activity of both  
252 Ibrutinib and Sotrastaurin, a Protein Kinase C inhibitor(29,30). *MYD88* mutations are frequent



253 in ABC DLBCL, L265P being the most prevalent variant (up to 29% in previous studies)(18).  
254 In our cohort, *MYD88* mutations were significantly enriched in ABC patients (28.4%,  
255  $FDR=4.7\times 10^{-3}$ ), with the L265P mutation present in 21% of ABC patients (Figures 3 and  
256 S5A). Of 17 ABC patients with *MYD88* L265P mutations, we found ten (58.8%) with  
257 associated *CD79B* mutations, who might respond more favorably to Ibrutinib treatment.  
258 *CD79B* mutations were identified in 10.7% of total patients, slightly less than previously  
259 reported, but were significantly enriched in ABC DLBCL (24.7%,  $FDR=3.2\times 10^{-5}$ ),  
260 corroborating previous studies(13,31). Of note, two (11.8%) *MYD88* L265P mutated ABC  
261 patients were *CARD11* mutated and two were *TNFAIP3* mutated, potentially diminishing the  
262 effect of Ibrutinib and Sotrastaurin, while six (35.3%) were *PRDMI* mutated (Figure S6).  
263 ABC cells have been shown to be addicted to IRF4 activity (32), responsible for  
264 Lenalidomide treatment response. 21 *IRF4* variants were discovered in 7.9% of patients  
265 (13.6% of ABC, 4.8% of GCB, 0% of “other” and 11.1% of PMBL) (Figure 1). Although the  
266 impact on expression of these mutations was not analyzed, only one *IRF4* variant was a stop-  
267 gain mutation, indicating that expression is most likely at least conserved. Most of the non-  
268 synonymous SNVs were clustered around amino acid positions 18 (two S18 variants and two  
269 G20 variants) and 99 (four C99R variants, SIFT scores=0) (Figure 4). Interestingly, *IRF4*  
270 mutations were almost equally frequent in PMBL and ABC in our cohort. Although IRF4  
271 addiction has not been demonstrated in PMBL, this predilection for *IRF4* mutations, added to  
272 the well-known high expression of IRF4 in this subtype, may suggest a potential role for  
273 Lenalidomide treatment in this subtype as well, calling for further investigation of these  
274 mutations’ effects.

275 Recently, small molecule inhibitors of PIM kinases have been developed and studied in  
276 DLBCL. In our cohort, *PIMI* mutations were significantly skewed toward ABC (33.3%,  
277  $FDR=3.3\times 10^{-5}$ ) in our cohort and represented 20.2% of ABC variants (Figures 2A, 3 and  
278 S5A). Of note, truncating *PIMI* variants were predominant in ABC patients (13 of 14  
279 identified). Interestingly, DLBCL sensitivity to PIM kinase inhibitors is not correlated with  
280 the level of PIM kinase expression (33), suggesting that even expression-modifying *PIMI*  
281 mutations might not necessarily hamper the efficacy of PIM1 inhibitors, although this remains  
282 to be verified. In any case, the high frequency of *PIMI* mutations in our cohort seems to  
283 justify the pre-screening of PIM kinase inhibitor candidate patients by *PIMI* sequencing, in  
284 order to identify those most likely to benefit from this targeted therapy.

285 The most highly mutated gene in GCB, *BCL2*(34), can notably be targeted by BH3-mimetics.  
286 Among our GCB cohort, *BCL2* was the third most frequently mutated gene (24.1%,

287 FDR=2.5x10<sup>-5</sup>) (Figures 3 and S5B), with mutations most frequently impacting the amino  
288 terminus and between the BH1 and BH3 domains. The BH3 domain was not impacted,  
289 indicating that BH3 mimetic activity would not be hampered in these patients (Figure S7).  
290 Targeting epigenetic changes is also being increasingly explored in the realm of tailored  
291 therapy, notably among the GCB subtype, justifying the inclusion of genes such as *EZH2*,  
292 *CREBBP*, *KMT2D* and *EP300* within the Lymphopanel. In our cohort, 18.1% of GCB patients  
293 were *EZH2* mutated, mostly at the Y641 hotspot, and *EZH2* mutations were enriched in GCB  
294 (FDR=1.8x10<sup>-3</sup>), as observed in previous studies, with no *EZH2* mutated ABC patients(13)  
295 (Figures 3 and S5B). Interestingly, one PMBL patient presented an *EZH2* Y641 mutation,  
296 adding to the debate of which DLBCL subtypes might benefit from *EZH2* inhibitor  
297 treatment(26). *CREBBP* mutations were identified in 31.3% of GCB patients and 6.2% of  
298 ABC patients, corroborating previous studies(17), but were also found in 24.2% of “other”  
299 and 11.1% of PMBL (Figures 1 and S5). *KMT2D* mutations, evenly distributed throughout the  
300 coding sequence, were identified in 40.9% of our cohort, with *KMT2D* being the most  
301 frequently mutated gene in all subtypes except PMBL (Figure 1). Of the 124 variants, 78 were  
302 truncating mutations, indicating bias toward loss of protein expression.  
303 Mutations of master regulators *TP53* and *MYC* showed no significant difference in subtype  
304 distribution in our cohort (Figure 3). *TP53* variants were present in 14.9% of total patients  
305 (slightly less than previously reported(13,35)) (Figure 1) and several recurrent variants were  
306 identified, including six R248 variants, shown to be loss of function mutants in DLBCL(35).  
307 As for *MYC*, we discovered mutations in 6.5% of total patients (4.9% of ABC, 9.6% of GCB,  
308 3% of “other” and 5.6% of PMBL). No mutations were identified at the known hotspots in  
309 Burkitt Lymphoma (BL), including S62 and T58.  
310 Finally, although frequent mutations are most enticing for therapeutic potential, rare  
311 mutations can also be potentially very effectively actionable. For instance, *BRAF* and  
312 *NOTCH1* mutations have been identified as rare but functionally relevant in DLBCL(13). In  
313 our cohort, we identified one patient with an activating V600E *BRAF* variant. As for  
314 *NOTCH1*, ten patients in our cohort presented mutations (Figure 1). *TCF3* was mutated in  
315 three patients, all of whom harbored a pathogenic N551K variant, which has shown altered  
316 DNA-binding specificity(36). The negative regulator of *TCF3*, *ID3*, was mutated in 4.7% of  
317 patients (Figure 1), with most variants affecting the HLH domain (Figure S7), potentially  
318 impairing its ability to inactivate *TCF3*, as shown in BL(36). *ID3* and *TCF3* mutations have  
319 recently been shown to be of comparable frequency in BL and DLBCL, highlighting the  
320 overlap between these two entities(37).

321

322 **The Lymphopanel highlights a unique PMBL mutational signature**

323 *STAT6* and *SOCS1* mutations were both very significantly enriched in PMBL (FDR=6.8x10<sup>-14</sup>  
324 and FDR=2.5x10<sup>-5</sup> respectively), with *STAT6* being the most frequently mutated gene in  
325 PMBL (72.2%, Figures 3 and S5C). All *STAT6* mutations were non-synonymous SNVs and  
326 recurrent variants were frequent among PMBL, including seven N417 variants (Figure S7).  
327 *SOCS1* mutations were present in 55.5% of PMBL patients, 30% of them harboring *SOCS1*  
328 inactivating mutations (Figure S5C). *TNFAIP3* mutations have also been frequently identified  
329 in PMBL (up to 36%)(38). We found *TNFAIP3* mutations to be significantly enriched in  
330 PMBL (61.1%, FDR=3.1x10<sup>-5</sup>), with all but one *TNFAIP3* mutated PMBL patient harboring  
331 truncating mutations (Figures 3 and S5C). The frequent *TNFAIP3* and *SOCS1* mutations in  
332 PMBL highlight the similarities between PMBL and cHL(38,39). Furthermore, no *MYD88*,  
333 *CD79B* or *CARD11* mutations were observed in PMBL patients (Figure 1), suggesting a  
334 PMBL dependence on *TNFAIP3* mutations for the constitutive activation of NFkB.  
335 Interestingly, significant *GNAI3* mutation enrichment was shown in PMBL rather than GCB  
336 in our cohort (50%, FDR=2.8x10<sup>-4</sup>) (Figures 3 and S5), and one third of *GNAI3* variants were  
337 potentially truncating mutations (Figure 4).

338 We discovered 46 *B2M* variants in 18.1% of total patients, significantly enriched in PMBL  
339 (50%, FDR=1.2x10<sup>-3</sup>) (Figures 1, 3 and S5C). Six of nine *B2M* mutated PMBL patients  
340 harbored truncating mutations and the remaining three presented pathogenic non-synonymous  
341 SNVs, two of which were a highly recurrent p.M1R variant predicted to affect the start codon  
342 (Figure 4). Recently, frequent *B2M* mutations leading to lack of protein expression were  
343 identified in cHL(9). Our similar findings in PMBL further highlight the molecular  
344 similarities between these two entities. *CD58* variants were also significantly enriched in  
345 PMBL (FDR=1.2x10<sup>-3</sup>) (Figures 3 and S5), with five of seven *CD58* mutated PMBL patients  
346 exhibiting truncating mutations, potentially leading to a lack of CD58 expression.  
347 Importantly, PD-1 blockade has recently shown promise as an effective treatment of relapsed  
348 or refractory cHL(40). Escape from T cell immunity via B2M and/or CD58 mutations could  
349 potentially affect the activity of PD-1 inhibitors in PMBL and cHL and warrants further  
350 investigation. Furthermore, we found 39 *CIITA* variants, enriched in PMBL as well (55.6% of  
351 PMBL versus 14.14% of total patients, FDR=3.1x10<sup>-5</sup>) (Figures 1, 3 and S5C). To our  
352 knowledge, this is the first study to identify truncating immunity pathway mutations as major  
353 in PMBL, although *CIITA/PDL1-2* translocations had been shown to impact PMBL  
354 survival(41).

355 *ITPKB*, *MFHAS1* and *XPO1* mutations were identified as significantly overrepresented in a  
356 relapsed/refractory DLBCL patient cohort(25), leading to their inclusion in the Lymphopanel.  
357 In our cohort, *ITPKB*, *MFHAS1* and *XPO1* mutations were all identified for the first time as  
358 being significantly enriched in PMBL patients (FDR=1.4x10<sup>-2</sup>, FDR=3.9x10<sup>-3</sup> and  
359 FDR=4.8x10<sup>-10</sup> respectively, Figure 3). The roles of *ITPKB* and *MFHAS1* in  
360 lymphomagenesis are not yet fully elucidated. *ITPKB* regulates B cell survival and function,  
361 and *ITPKB* deficiency leads to B cell antigen presentation unresponsiveness and decreased B  
362 cell survival, associated with specific overexpression of proapoptotic Bim protein(42). We  
363 found 48 *ITPKB* variants, present in 14% of total patients (Figures 1 and S5). *ITPKB*  
364 mutations were uniformly distributed along the protein sequence, excepting the seemingly  
365 conserved catalytic IPK domain, affected by only two non-synonymous SNVs (Figure 4).  
366 *MFHAS1* is a potential oncogene with highly conserved leucine-rich tandem repeats, isolated  
367 from a minimal common amplified region in malignant fibrous histiocytomas at 8p23.1.  
368 *MFHAS1* is also a target gene for chromosomal translocation t(8;14)(p23.1;q21) in B-cell  
369 lymphoma cell lines and has been shown to be tumorigenic in nude mice(43). In our cohort,  
370 *MFHAS1* variants were identified in 7.9% of total patients, and were distributed  
371 homogeneously along the coding sequence, with no recurrent mutations identified (Figure 4).  
372 Although the role of these potential oncogenes in lymphomagenesis is still unclear, their high  
373 mutation frequency in PMBL especially warrants further investigation.  
374 As for *XPO1*, it encodes CRM1, an exporter of several tumor suppressor proteins (TSPs).  
375 Cytoplasmic export of TSPs renders them inactive, indicating that *XPO1* acts as a proto-  
376 oncogene. *XPO1* mutations were present in 4.7% of total patients (1.2% of ABC, 1.2% of  
377 GCB, 3% of “other” and 38.9% of PMBL) (Figures 1 and S5). The mutation identified is  
378 recurrent, with all variants affecting E571 (Figure 4), as previously found in rare CLL cases  
379 (<5%)(44). Importantly, Selective Inhibitors of Nuclear Export (SINEs), have been shown to  
380 effectively target CRM1 and retain TSPs in the nucleus(45). Given the frequency of *XPO1*  
381 E571 mutations in PMBL, further investigation concerning their impact on response to SINEs  
382 is warranted (Jardin et al, under review).

383

#### 384 **Identification of gene mutations correlated with clinical characteristics and prognosis**

385 We tested each gene in the Lymphopanel for correlation with clinical characteristics including  
386 age, stage, IPI, bone marrow involvement and presence of bulky disease.

387 *CD79B*, *KMT2D* and *MYD88* mutations were significantly correlated with age (Figure S8).

388 For *CD79B* and *MYD88* mutations, enriched in ABC, this might be linked to the significantly

389 higher age of ABC patients ( $p=1.01 \times 10^{-7}$  in our cohort)(46). As for *KMT2D* mutations,  
390 homogenous among subtypes, this result potentially suggests an accumulation of passenger-  
391 type mutations, particularly visible in this long gene (16.6 kb sequenced). *MYD88* mutations  
392 were also significantly correlated with higher IPI (FDR=0.04).

393 On the other hand, PMBL patients were significantly younger ( $p=1.21 \times 10^{-8}$ ) and the large  
394 majority of genes whose mutations were significantly enriched in PMBL were also correlated  
395 with younger age (Figure S8). In addition, *B2M* and immunity pathway mutations were  
396 significantly inversely correlated with Ann Arbor stage (FDR= $3.2 \times 10^{-3}$  and FDR=0.045  
397 respectively), and *B2M* and *STAT6* mutations were significantly inversely correlated with IPI  
398 (FDR= $5.5 \times 10^{-4}$  and FDR=0.026 respectively).

399 As expected, OS and PFS decreased with increasing IPI and ABC patients displayed  
400 significantly worse OS and PFS than GCB patients in the R-CHOP treatment group  
401 ( $p=1.9 \times 10^{-2}$  and  $p=6.6 \times 10^{-3}$  respectively) (Figure S9). Of note, PMBL patients in our cohort  
402 did not present the favorable prognosis typically associated with this subtype(47), perhaps due  
403 to the lack of dedicated treatment.

404 Prognostic impact was assessed for all Lymphopanel genes and subtypes, among patients  
405 treated with R-chemotherapy, separated into an R-CHOP group and an R-ACVBP group.  
406 Analyses over all subtypes were performed using multivariate Cox tests, while analyses of  
407 each subtype individually were performed using log-rank tests. In both cases, independent  
408 corrections of p-values were performed for OS and PFS. Gene mutations harboring prognostic  
409 impacts with uncorrected p-values < 0.05 are highlighted in Table 1 and Figure S10, and all  
410 survival analyses are presented in Table S7. We set an FDR threshold of 0.05 to detect gene  
411 mutations with significant prognostic impacts.

412 Among ABC DLBCL treated with R-CHOP, *TNFAIP3* mutations were significantly  
413 associated with lower OS (FDR= $7.86 \times 10^{-4}$ ) and PFS (FDR= $3.04 \times 10^{-2}$ ), while *GNAI3*  
414 mutations were significantly associated with lower PFS (FDR= $3.04 \times 10^{-2}$ ), (Figure 5). Of  
415 note, the poor prognostic impact of these mutations was not observed in patients treated with  
416 R-ACVBP, which, for *TNFAIP3* at least, might be linked to the improved survival observed  
417 in younger patients with DLBCL treated with R-ACVBP(48). These findings need to be  
418 confirmed in an independent cohort of patients, given the small sample size.

419 Unfortunately, the prognostic impact of *TNFAIP3* mutations in PMBL patients could not be  
420 assessed due to the existence of only one WT *TNFAIP3* PMBL patient treated with R-CHOP  
421 (Table S7). Moreover, previously described prognostic impacts of *TP53*, *FOXO1* or *MYD88*

422 mutations were not confirmed(35,49,50), although the impact of specific deleterious  
423 mutations was not analyzed on its own.

424

425 In conclusion, NGS is increasingly accessible in academic research settings, but its  
426 possibilities in routine clinical settings have yet to be thoroughly harnessed. This study has  
427 shown that, by using a restricted set of genes, not only can well-known mutations be tracked,  
428 but novel or rare mutations can also be identified and potentially targeted as well. Detailing  
429 subtype-enriched gene and pathway mutations is critical for optimal understanding and  
430 treatment of DLBCL: here, we have added to the field's current knowledge of the genetic  
431 heterogeneity between DLBCL subtypes, and identified novel clinical and prognostic  
432 correlations, which might also have an impact on therapeutic decisions. NGS with a  
433 consensus targeted panel could contribute valuable information to multidisciplinary meetings  
434 and constitute a molecular breakthrough in the way treatment decisions are made, by  
435 providing personalized mutational profiles of actionable targets. Furthermore, although this  
436 study was performed on frozen tumor samples, which are of limited availability, the  
437 Lymphopanel was designed to be effective in FFPE samples as well, further cementing its  
438 capacity to play an essential role in clinical disease management. In addition, our team has  
439 recently demonstrated that Lymphopanel NGS can be successfully performed using plasma-  
440 extracted cell-free circulating DNA, highlighting its feasibility and potential role in the  
441 monitoring of DLBCL(51). Taken together, these results serve as proof-of-principle that NGS  
442 with a consensus gene panel can alter the manner in which DLBCL patients are subclassified  
443 and treated.

444

#### 445 **Acknowledgements**

446 This study was funded by grants from the Institut National du Cancer (INCA). We thank  
447 Marie-Hélène Delfau, Camille Laurent and Loïc Ysebaert for their critical review.

448

#### 449 **Contributors**

450 SD, P-JV, SM, EB, PB, PR, J-PJ, MF, GS, HT, KL and FJ contributed to the conception and  
451 design of the study. TJM, FD, TF, CH, TL, CC-B, JB, TP, DC, BF, BC, RD, FP, AB, MA and  
452 NK collected clinical data. EB and CM performed NGS experiments. PP performed GEP  
453 experiments. SD, P-JV and SM performed statistical analyses and contributed to data  
454 interpretation. P-JV and SM developed the bioinformatics pipeline necessary for data

455 analysis. SD wrote the paper and all authors provided critical revisions and gave final  
456 approval to submit for publication.

457

## 458 **Figure and Table Legends**

459 **Figure 1: Mutation frequencies in the total cohort and by subtype.** Mutation frequencies  
460 in the total cohort are represented as a stacked bar chart. The number at the top of each bar  
461 represents the mutation frequency of the indicated gene in the total cohort. Bars are  
462 subdivided by DLBCL subtype, with segments proportional to mutation frequency.  
463 Potentially actionable targets are highlighted by indicating the appropriate class of molecule,  
464 which could potentially be used.

465 **Figure 2: Mutation pathway heterogeneity among DLBCL subtypes.** Pie-chart  
466 representations of mutation frequencies represented by pathway are represented for ABC (A),  
467 GCB (B), PMBL (C) and Other (D) subtypes. Mutation frequencies per gene and per subtype  
468 are shown here as the percentage of the total number of variants. Genes were grouped into 8  
469 specific pathways: Immunity (*CIITA*, *B2M*, *TNFRSF14* and *CD58*), NOTCH (*NOTCH1* and  
470 *NOTCH2*), Apoptosis/Cell Cycle (*MFHAS1*, *XPO1*, *MYC*, *CDKN2A/B*, *FOXO1*, *TP53*,  
471 *GNA13* and *BCL2*), NFkB (*TNFAIP3*, *MYD88*, *PIM1*, *CARD11*, *IRF4* and *PRDM1*),  
472 Epigenetic Regulation (*EZH2*, *KMT2D*, *EP300*, *MEF2B* and *CREBBP*), MAP Kinases  
473 (*BRAF*), JAK-STAT (*SOCS1* and *STAT6*), and BCR (*CD79A/B*, *ITPKB*, *TCF3* and *ID3*).

474 **Figure 3: Heatmap of mutation frequencies by subtype.** Mutation frequencies in each  
475 subtype are indicated for each gene of the Lymphopanel. Mutation frequencies per gene and  
476 per subtype are shown here as the percentage of the total number of patients. Statistical  
477 significance of gene mutation enrichment in a given subtype is indicated by the FDR column.  
478 The horizontal line separates genes with  $FDR < 0.05$ , considered significant, from genes with  
479  $FDR \geq 0.05$ .

480 **Figure 4. Mutations at the protein level.** Protein representations were rendered using  
481 domains imported from the Pfam database (green). Exons were located using the CCDS  
482 database and were numbered automatically, not counting non-coding and spliced exons.  
483 Substitutions are depicted as diamonds, insertions as triangles and deletions as rectangles  
484 whose length varies according to the deletion size. A color code is also applied, with blue  
485 indicating non-synonymous substitutions or non-frameshift insertions, yellow indicating  
486 insertions and frameshift deletions, and pink indicating stop-gain and stop-loss mutations.  
487 Blue shading indicates sequenced regions.

488 **Figure 5: *TNFAIP3* and *GNA13* mutations are linked to worse prognosis in R-CHOP-**  
489 **treated ABC patients.** Survival analysis was performed on ABC patients treated with R-  
490 CHOP according to the presence or absence of *TNFAIP3* mutation (A, B) or *GNA13* mutation  
491 (C, D). Both *TNFAIP3* and *GNA13* mutations are associated with significantly less favorable  
492 prognosis in R-CHOP treated ABC patients.

493 **Table 1. Prognostic impact of gene mutations among the Lymphopanel.** Gene mutations  
494 with a tendency toward prognostic impact (uncorrected  $p < 0.05$ ) are indicated, for OS and/or  
495 PFS. The subtype and treatment cohort in which a prognostic impact was identified is shown,  
496 as is the number of mutant and WT patients for each survival analysis. HR column indicates  
497 Hazard Ratio. Bold type highlights significant FDR values.

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<b>Gene</b>	<b>Subtype</b>	<b>Cohort</b>	<b>Type</b>	<b>Mutant (n)</b>	<b>WT (n)</b>	<b>HR</b>	<b>p</b>	<b>FDR</b>
<i>TNFAIP3</i>	ABC	R-CHOP	OS	6	48	9.04 [2.82-28.98]	9.03E-06	<b>7.86E-04</b>
<i>TNFAIP3</i>	ABC	R-CHOP	PFS	6	48	5.59 [1.91-16.39]	4.29E-04	<b>3.04E-02</b>
<i>GNA13</i>	ABC	R-CHOP	PFS	5	49	5.89 [1.86-18.64]	6.47E-04	<b>3.04E-02</b>
<i>B2M</i>	all	R-CHOP	PFS	16	100	0.12 [0.03-0.55]	6.24E-03	3.25E-01
<i>B2M</i>	all	R-CHOP	OS	16	100	0.11 [0.01-0.82]	3.13E-02	5.43E-01
<i>IRF4</i>	all	R-CHOP	OS	10	106	2.53 [1.10-5.87]	2.98E-02	5.43E-01
<i>CD79B</i>	all	R-CHOP	OS	19	97	0.31 [0.11-0.90]	3.13E-02	5.43E-01
<i>CD79B</i>	all	R-CHOP	PFS	19	97	0.29 [0.10-0.83]	2.15E-02	5.58E-01
<i>CD58</i>	all	R-CHOP	PFS	12	104	2.36 [1.07-5.20]	3.27E-02	5.67E-01
<i>GNA13</i>	ABC	R-CHOP	OS	5	49	3.68 [1.21-11.15]	1.36E-02	5.94E-01
<i>FOXO1</i>	GC	R-CHOP	OS	5	35	3.87 [1.00-15.07]	3.53E-02	7.72E-01
<i>CD79B</i>	ABC	R-CHOP	OS	16	38	0.35 [0.12-1.02]	4.44E-02	7.72E-01
<i>CD79B</i>	ABC	R-CHOP	PFS	16	38	0.33 [0.11-0.95]	3.07E-02	8.30E-01

**Table 1. Prognostic impacts of gene mutations among the Lymphopanel**

Figure 1

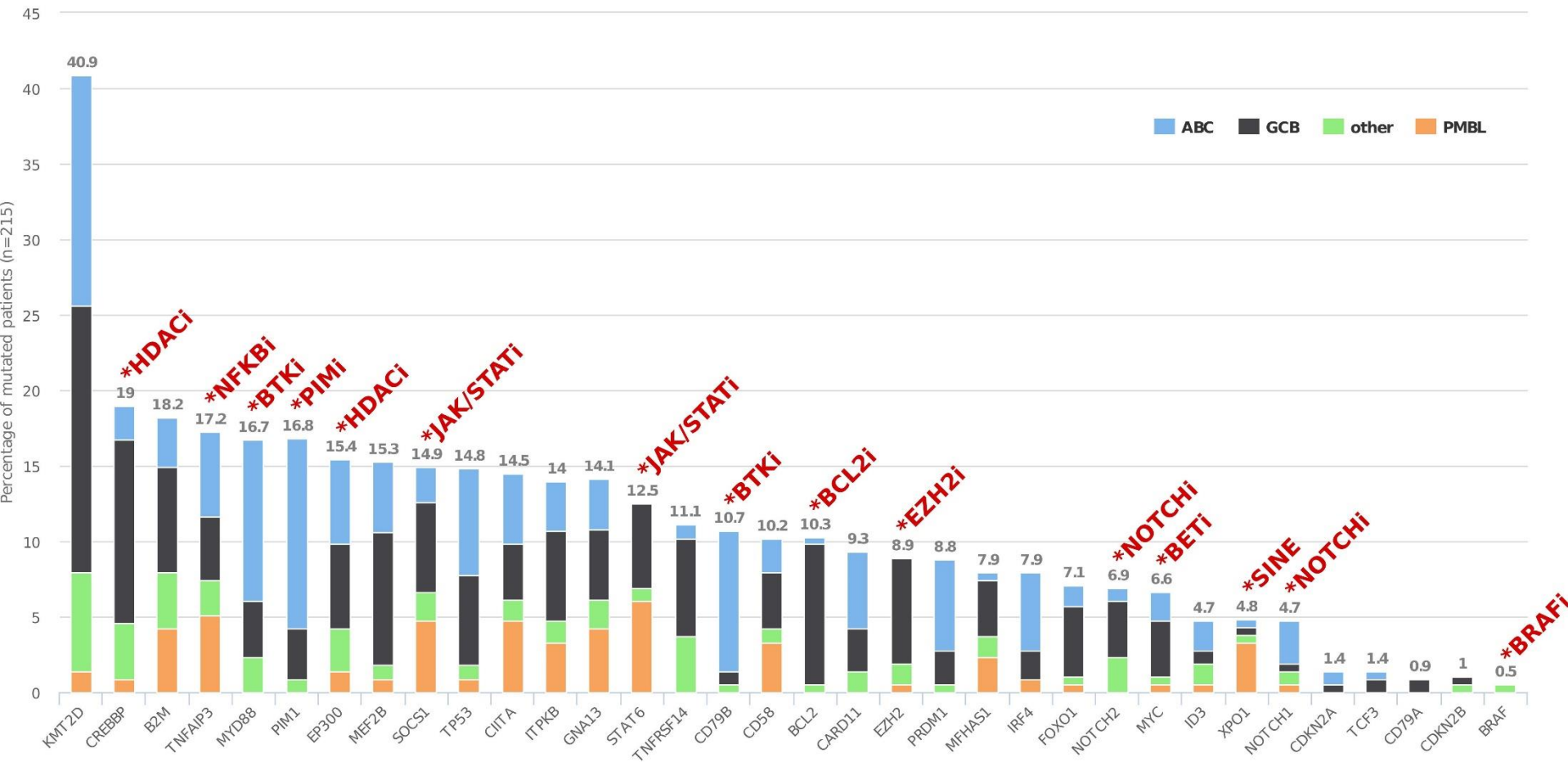


Fig. 2

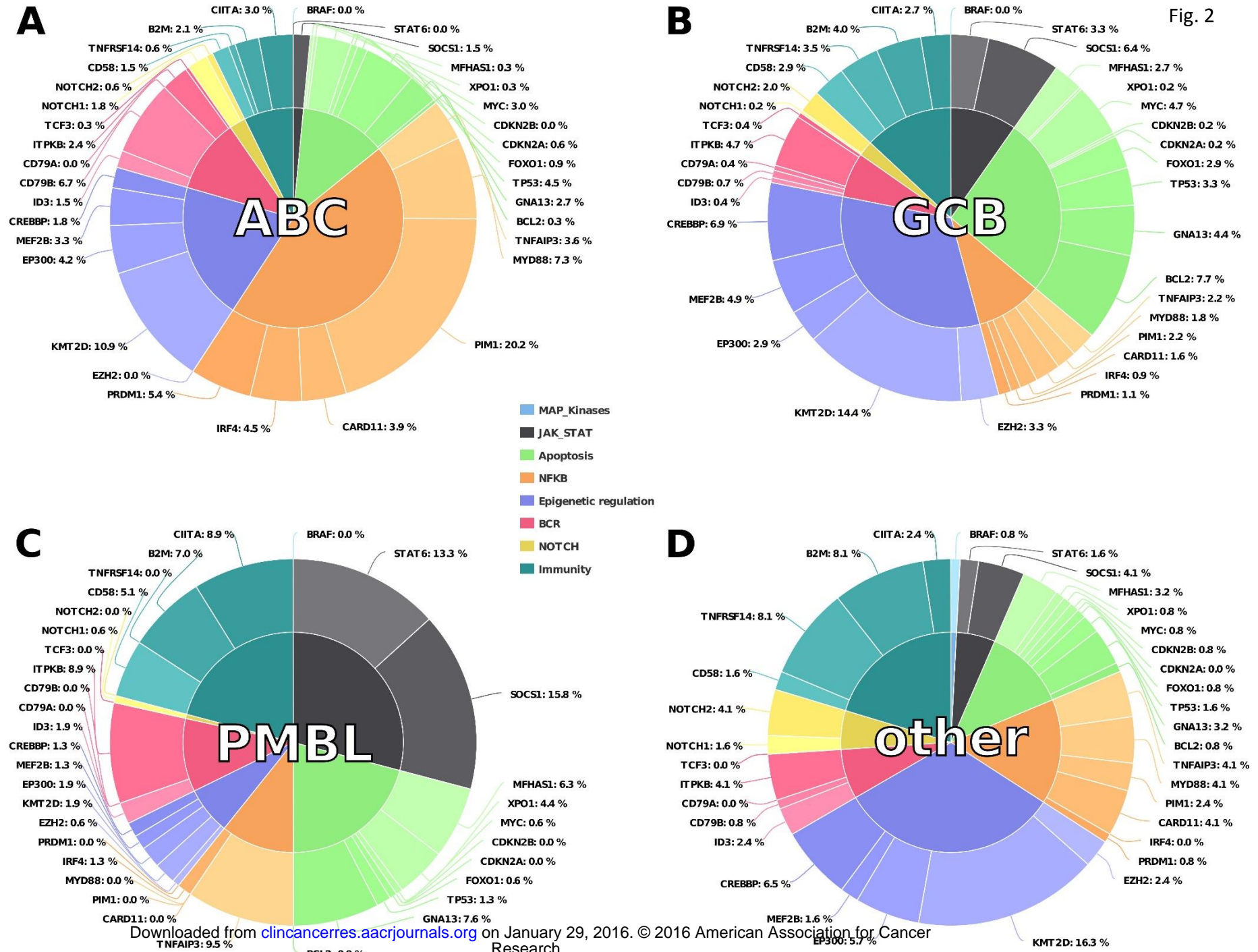


Figure 3

	<b>ABC</b> n = 81	<b>GCB</b> n = 83	<b>PMBL</b> n = 18	<b>other</b> n = 33	FDR
STAT6	0%	14%	72%	6%	<b>6.8e-14</b>
XPO1	1%	1%	39%	3%	<b>4.8e-10</b>
SOCS1	6%	16%	56%	12%	<b>2.5e-05</b>
BCL2	1%	24%	0%	3%	<b>2.5e-05</b>
CIITA	12%	10%	56%	9%	<b>3.1e-05</b>
TNFAIP3	15%	11%	61%	15%	<b>3.1e-05</b>
CD79B	25%	2%	0%	3%	<b>3.2e-05</b>
PIM1	33%	8%	0%	6%	<b>3.3e-05</b>
GNA13	9%	12%	50%	12%	<b>2.8e-04</b>
CD58	6%	10%	39%	6%	<b>1.2e-03</b>
CREBBP	6%	31%	11%	24%	<b>1.2e-03</b>
B2M	9%	18%	50%	24%	<b>1.2e-03</b>
EZH2	0%	18%	6%	9%	<b>1.8e-03</b>
TNFRSF14	2%	17%	0%	24%	<b>1.8e-03</b>
MFHAS1	1%	10%	28%	9%	<b>3.9e-03</b>
MYD88	28%	10%	0%	15%	<b>4.7e-03</b>
ITPKB	9%	16%	39%	9%	<b>1.4e-02</b>
PRDM1	16%	6%	0%	3%	5.1e-02
NOTCH2	2%	10%	0%	15%	7.6e-02
IRF4	14%	5%	11%	0%	8.7e-02
MEF2B	12%	23%	11%	6%	1.4e-01
BRAF	0%	0%	0%	3%	2.1e-01
FOXO1	4%	12%	6%	3%	2.1e-01
KMT2D	41%	46%	17%	42%	2.2e-01
CARD11	14%	7%	0%	9%	3.5e-01
NOTCH1	7%	1%	6%	6%	3.7e-01
CD79A	0%	2%	0%	0%	4.5e-01
TP53	19%	16%	11%	6%	4.6e-01
CDKN2B	0%	1%	0%	3%	5.4e-01
ID3	5%	2%	6%	9%	5.5e-01
MYC	5%	10%	6%	3%	5.5e-01
CDKN2A	2%	1%	0%	0%	7.4e-01
TCF3	1%	2%	0%	0%	7.4e-01
EP300	15%	14%	17%	18%	9.6e-01

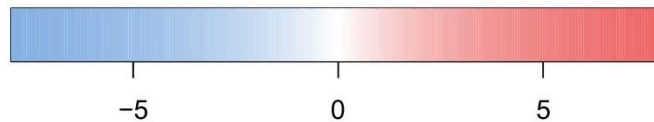




Figure 4

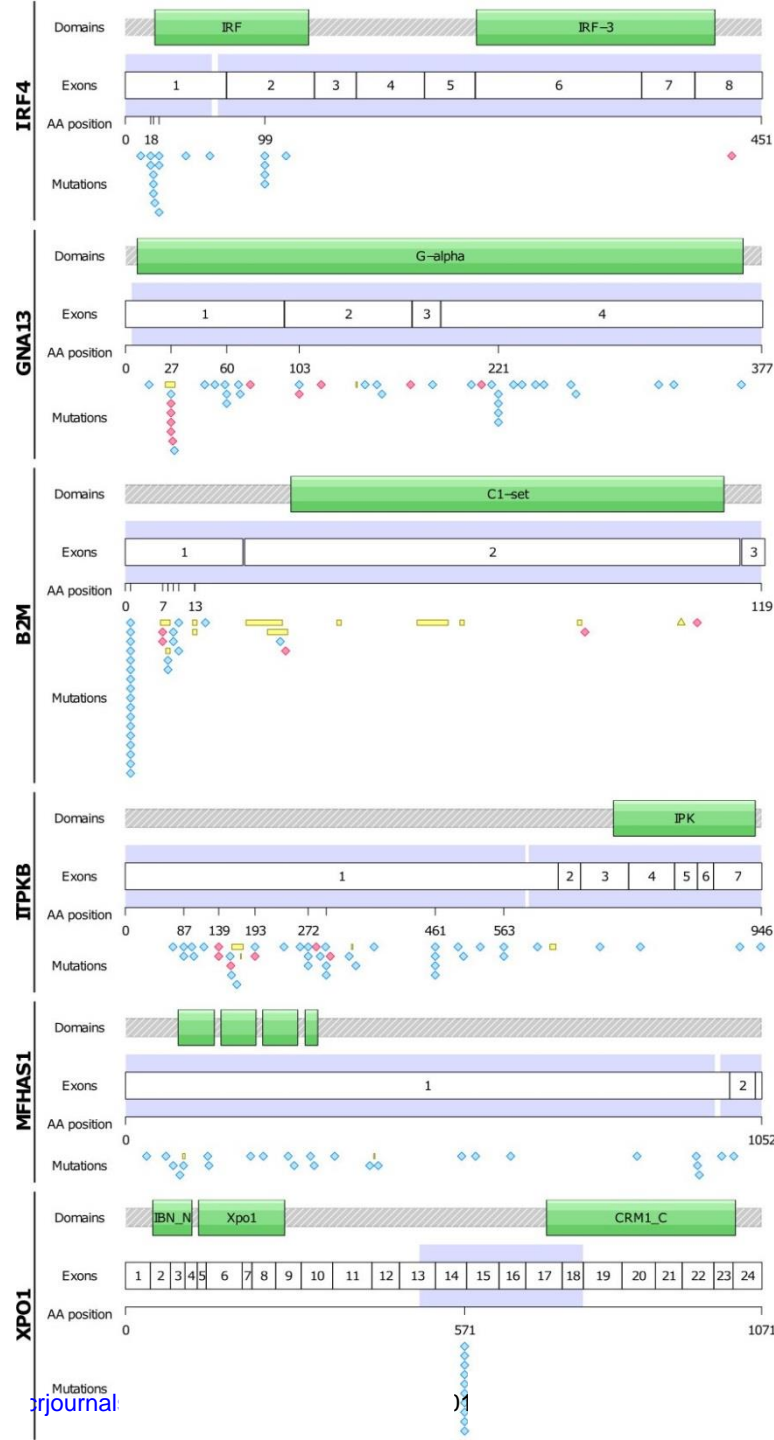


Figure 5

