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Longitudinal expression profiling identifies a poor risk subset of patients with ABC-type Diffuse Large B Cell Lymphoma

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Abstract:

Despite the effectiveness of immuno-chemotherapy, 40% of patients with diffuse large B-cell lymphoma (DLBCL) experience relapse or refractory disease. Longitudinal studies have previously focused on the mutational landscape of relapse but falling short of providing a consistent relapse-specific genetic signature. In our study, we have focussed attention on the changes in gene expression profile accompanying DLBCL relapse using archival paired diagnostic/relapse specimens from 38 de novo DLBCL patients. Cell of origin remained stable from diagnosis to relapse in 84% of patients, with only a single patient showing COO switching from ABC to GCB. Analysis of the transcriptomic changes that occur following relapse suggest ABC and GCB relapses are mediated via different mechanisms. We developed a 30-gene discriminator for ABC-DLBCLs derived from relapse-associated genes, that defined clinically distinct high and low risk subgroups in ABC-DLBCLs at diagnosis in datasets comprising both population-based and clinical trial cohorts. This signature also identified a population of <60-year-old patients with superior PFS and OS treated with Ibrutinib-R-CHOP as part of the PHOENIX trial. Altogether this new signature adds to the existing toolkit of putative genetic predictors now available in DLBCL that can be readily assessed as part of prospective clinical trials.

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 3 **Large B Cell Lymphoma**

4 **Running Title (50):**

5 **Longitudinal expression profiling of rrDLBCL**

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39 **Competing Interests:**

40 K.K. is an employee and shareholder of Roche

41 J.F. has provided consultancy and received funding from Epizyme

42 D.W.S, L.M.R have IP rights to the Lymph2Cx assay

43 D.W.S. has provided consultancy to Abbvie, AstraZeneca, Celgene, Janssen and Incyte and
44 has received research funding from Janssen, NanoString Technology and Roche/Genentech.

45

46 **Abstract**

47 Despite the effectiveness of immuno-chemotherapy, 40% of patients with diffuse large B-cell
48 lymphoma (DLBCL) experience relapse or refractory disease. Longitudinal studies have previously
49 focused on the mutational landscape of relapse but fell short of providing a consistent relapse-
50 specific genetic signature. In our study, we have focussed attention on the changes in gene
51 expression profile accompanying DLBCL relapse using archival paired diagnostic/relapse specimens
52 from 38 de novo DLBCL patients. Cell of origin remained stable from diagnosis to relapse in 80% of
53 patients, with only a single patient showing COO switching from ABC to GCB. Analysis of the
54 transcriptomic changes that occur following relapse suggest ABC and GCB relapses are mediated via
55 different mechanisms. We developed a 30-gene discriminator for ABC-DLBCLs derived from relapse-
56 associated genes, that defined clinically distinct high and low risk subgroups in ABC-DLBCLs at
57 diagnosis in datasets comprising both population-based and clinical trial cohorts. This signature also
58 identified a population of <60-year-old patients with superior PFS and OS treated with Ibrutinib-R-
59 CHOP as part of the PHOENIX trial. Altogether this new signature adds to the existing toolkit of
60 putative genetic predictors now available in DLBCL that can be readily assessed as part of prospective
61 clinical trials.

62 **Key Points (140 each):**

63 Cell of origin is stable between diagnosis and relapse

64 A 30 gene panel of relapse associated genes was able to stratify ABC patient survival at diagnosis.

65

66 **Introduction**

67 Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease encompassing multiple molecular
68 and biological subtypes. Although potentially curable with immuno-chemotherapy, up to 40% of
69 patients will experience relapsed or refractory disease ^{1,2}. The current standard of care for these
70 lymphomas has not changed in the past 2 decades, and efforts are turning to identification of sub-
71 groups of DLBCL that may demonstrate preferential response to existing or novel therapies ^{3,4}. Early
72 work focused on DLBCL at diagnosis, using gene expression profiling to delineate the “cell-of origin”
73 (COO) classification system (Germinal Centre B-cell-like (GCB), Activated B-cell-like (ABC) and
74 unclassified) with ABC tumours being linked to poorer outcome ⁵. However, attempts to utilise
75 molecular analyses to tailor treatment, and specifically to develop alternative R-CHOP (Rituximab,
76 Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone) regimens to mitigate the poorer outcome
77 of patients in the ABC-DLBCL subgroup, have not, to date, led to significant improvements ⁶⁻⁹.

78
79 More recently, attempts to refine the taxonomy of DLBCL through integrative genomic analysis have
80 demonstrated additional heterogeneity not captured by the previous COO classification ¹⁰⁻¹⁴. This has
81 led to the growing realisation that DLBCL encompasses a number of biological entities with distinct
82 oncogenic mechanisms, requiring a more sophisticated approach to patient management and trial
83 design. To date, these studies have predominantly focused on analysing single tumour biopsies at
84 diagnosis, with our understanding of the pre-programmed or acquired mechanisms underpinning
85 relapsed disease hindered by the limited availability of sequential biopsy samples. The majority of
86 longitudinal studies published thus far have focussed on genetic changes between the diagnostic and
87 relapse tumour, providing important confirmation of the clonal relationship between diagnosis and
88 relapse, and describing recurrent relapse-associated genetic aberrations, but fell short of providing a
89 consistent relapse-specific genetic signature ¹⁵⁻²² (**Supplemental Table 1**). In this study, we sought to
90 utilise gene expression profiling in paired diagnostic and relapse tumours to further understand the
91 mechanisms underpinning treatment failure following immuno-chemotherapy. Using these data, we

92 demonstrate the stability of COO at relapse in the majority of cases and identify a novel relapse-
93 associated gene expression signature that reliably discriminated two distinct outcome groups within
94 the ABC type of DLBCL at diagnosis.

95

96 **Methods**

97 **Patient Cohort**

98 Ethical approval was obtained from the London Research Ethics Committee (LREC) of the East
99 London and the City Health authority (10/H0704/65 and 06/Q0605/69). Written consent was
100 obtained for the use of specimens for research purposes and samples from collaborating centres had
101 local ethical approval. Paired diagnosis/relapse DLBCL biopsies were collated from 38 patients across
102 5 centres in the UK. All patients were treated with standard first-line rituximab-based immuno-
103 chemotherapy (e.g. R-CHOP) and achieved either a partial or complete remission (**Figure 1A, Table**
104 **1**). COO was determined using the Lymph2Cx assay on the NanoString platform ²³ or the DLBCL
105 Automatic Classifier ²⁴ and all biopsies had $\geq 19\%$ total B cell content, as estimated by CIBERSORT ²⁵.
106 Thirty-four biopsies were nodal (15 diagnosis, 19 relapse) and 42 extranodal (23 diagnosis and 19
107 relapse). The site of the biopsy was identical at diagnosis and relapse for 20 cases (8 nodal, 12
108 extranodal).

109

110 **Gene Expression Analysis**

111 Gene expression profiling (GEP) of FFPE samples was carried out using the Ion Ampliseq™ Human
112 Gene Expression array, consisting of 20,802 genes. Poorly captured genes (0 reads in $\geq 1/3$ of the
113 cohort) were removed, leaving 15,457 genes. Raw read counts were normalised to \log_2 count per
114 million (CPM). Differential expression between matched relapse and diagnostic samples, and gene
115 set enrichment analysis ²⁶ were subsequently performed. The list of differentially expressed (DE)
116 genes were selected for the following gene signature discovery using publicly available datasets. The

117 expression data and sample information for the rrDLBCL cohort are available from GEO accession
118 GSE193566.

119

120 **Derivation of a prognostic gene panel**

121 Relapse associated genes found within our paired cohort ($p < 0.05$) were used in conjunction with
122 the Prediction Analysis of Microarrays²⁷ (PAM) algorithm to define a survival signature for DLBCL.
123 The expression of these genes within a cohort of 262 GCB and 249 ABC diagnostic DLBCL patients¹⁴
124 (called the Reddy cohort hereafter) was used to train the PAM model. For the validation of the
125 resulting gene signatures, a linear predictor model was constructed based on the prognostic value of
126 each gene in the training dataset and the expression value in the validation dataset. This predictor
127 score was used to stratify patients in three independent GEP cohorts: the REMoDL-B clinical trial⁷,
128 the LLMPP (Lymphoma/Leukemia Molecular Profiling Project series²⁸ and the Haematological
129 Malignancy Research Network (HMRN) population cohort²⁹ (hereafter referred to as the REMoDL-B,
130 LLMPP, and HMRN cohorts, respectively). All survival analyses were performed using the Cox
131 Proportional Hazards Model in R.

132

133 See supplemental methods for a full description of the methods.

134

135 **Results**

136 **COO is stable between diagnosis and relapse**

137 The longitudinal series included 38 paired diagnostic-relapsed DLBCLs (**Figure 1A, Table 1**), all treated
138 at diagnosis with R-CHOP or R-CHOP-like regimens. Cell of origin (COO) calling was successfully
139 completed in both biopsies for 35 cases. COO was stable across 28 patients (80%) and corresponded
140 to 17 ABC-ABC and 11 GCB-GCB pairs, with 2 further cases being unclassified (UNC) at both
141 timepoints (**Figure 1B**). Discordant COO was a feature of just 5 cases (1 ABC-GCB, 2 ABC-UNC, 1 GCB-
142 UNC, 1 UNC-ABC) with a single example of an ABC-GCB transition, suggesting that changes in DLBCL

143 trajectory at relapse, while reported in the literature³⁰, are uncommon. The median time to relapse
144 was 1.75 years, with 22 patients (58%) relapsing within two years. In two cases (1 GCB-GCB and 1
145 ABC-ABC) relapse occurred after more than 10 years (10.2 years and 13.9 years respectively).

146

147 **Deregulated gene expression between diagnosis and relapse**

148 We interrogated whole-transcriptome GEP data from all 76 biopsies with the aim of identifying
149 changes in gene expression associated with DLBCL relapse. Principal component analysis (PCA) based
150 on the full set of profiled genes (n=15,457) did not reveal distinct clustering of the diagnostic or
151 relapse samples (**Figure 1C**). There was no consistent pattern observed in the PCA values within the
152 individual pairs or based on the location of biopsies, nodal/extra-nodal disease, or time to relapse
153 (**Supplemental Figure 1A-C**). As expected, GEP profiles of the samples showed association based on
154 their COO (**Figure 1C**) where differential expression (DE) analyses of the ABC (n = 17) and GCB (n =
155 11) pairs identified unique sets of genes associated with relapse, based on COO (< 4% overlapping DE
156 genes, limma analysis $p < 0.05$) (**Supplemental Figure 1**). This was also supported by gene set
157 enrichment analysis (GSEA) where chromosome maintenance, DNA repair, and rRNA processing were
158 among the top upregulated pathways (FDR < 0.1) in the ABC-ABC series in comparison to adaptive
159 immunity, cytokine signalling and antigen processing and presentation signatures which were unique
160 to GCB-GCB pairs (**Figure 1D-E & Supplemental Tables 2-3**).

161

162 **A 30 gene outcome predictor in ABC DLBCL**

163 We postulated that the expression of these relapse associated genes might hold some prognostic
164 significance in a diagnostic cohort. To this end the PAM algorithm²⁷ was used to interrogate a total of
165 796 and 387 DE genes ($p < 0.05$) from our ABC and GCB diagnostic-relapse signatures respectively, in
166 the Reddy series of 262 GCB and 249 diagnostic ABC DLBCLs. This analysis identified a 30-gene
167 signature that separated ABC patients into 136 low and 113 high-risk cases with significantly different
168 overall survival (Hazard Ratio (HR)=1.89, 95% CI=1.26-2.83; log-rank $p=0.0017$; **Figure 1F**). The

169 majority of the genes in this panel have not previously been implicated in DLBCL pathogenesis,
170 although notable exceptions included *MYC* and *TNFRSF9*, with *MYC* one of five genes demonstrating
171 significant single-gene clinical association, inversely correlated with overall survival ($p < 0.05$; **Figure**
172 **1F**). STRING analysis of these 30 genes identified 7 highly interconnected clusters, with *MYC* at the
173 centre of this protein interaction network (**Supplementary Figure 5, Supplementary Table 6**). In
174 contrast to ABC patients, there was no equivalent predictor detected using PAM in the corresponding
175 set of GCB cases. Attempts to define a response signature using the Reddy cohort without the prior
176 enrichment of relapse associated genes were unsuccessful.

177

178 **Validation of the 30-gene ABC predictor in 3 independent DLBCL series**

179 The reproducibility of this 30 gene outcome predictor was evaluated in 3 separate DLBCL cohorts
180 (REMoDL-B and HMRN – both with RNA profiling achieved using the cDNA-mediated Annealing,
181 Selection, extension and Ligation (DASL) assay, and LLMP – RNA profiling from an Affymetrix
182 microarray chip ^{7,28,29}), all treated with R-CHOP (R-CHOP + bortezomib in 126 patients from the
183 REMoDL-B cohort) at diagnosis and comprising 504 ABC cases in total. We evaluated each series
184 separately. Within each cohort, a linear predictor score was calculated for each patient, based on the
185 summation of the expression of 29 or 30 genes (as not all genes were represented on each platform),
186 weighted by their beta coefficients from the training dataset (**Supplemental Table 4**). These linear
187 predictors were standardised using a Z-transformation and each cohort was subdivided into high
188 (standardized linear predictor >0) and low (standardized linear predictor <0) scoring risk groups (see
189 Supplemental Methods). Analysis of the cause of deaths in the HMRN cohort shows that patients
190 with lymphoma associated deaths had a significantly shorter follow up time than patients who died
191 from other causes (*Wilcoxon rank sum* $p < 0.001$, **Supplemental Figure 2D**). Moreover, it was notable
192 that non-lymphoma related deaths increased significantly from 3 years in this series and so we
193 restricted our analysis of overall survival accordingly.

194

195 The algorithm stratified the 255 ABC REMoDL-B cases, into 108 low and 147 high-risk cases (three
196 year OS; HR=2.04, 95% CI=1.073-3.875; $p=0.026$; **Figure 2A**), the LLMPP series of 93 ABC cases into
197 44 low and 49 high-risk cases (three year OS; HR=2.3, 95% CI=1.154-4.565; $p=0.015$; **Figure 2B**) and a
198 UK population-based cohort (HMRN) of 156 ABC cases, into 72 low and 84 high-risk cases (three year
199 OS; HR=1.93, 95% CI=1.06-3.522; $p=0.029$; **Figure 2C**). Across all three cohorts, patients with high
200 linear predictor scores (high-risk) showed significant reduction in survival at three years. When later
201 events were included, both the REMoDL-B and LLMPP data showed similar results (OS HR=2.11, 95%
202 CI=1.115-3.993; $p=0.019$ & HR=2.17, 95% CI=1.109-4.242; $p=0.02$; **Supplemental Figure 2A-B**
203 respectively), whilst the HMRN cohort showed a trend for reduced survival in the high-risk group,
204 (HR=1.39, 95% CI=0.917-2.106; $p=0.12$; **Supplemental Figure 2C**) and we have reasoned that the
205 performance of the discriminator may reflect the number of non-lymphoma related deaths in this
206 population-based cohort.

207

208 We restricted our multivariate analysis to the REMoDL-B and LLMPP series, accounting for patient
209 age, gender, IPI and stage (where available), and the high-scoring group remained associated with
210 poorer OS (HR=1.95, Wald test $p=0.042$ for REMoDL-B; HR=2.19, $p=0.023$ for LLMPP; **Supplemental**
211 **Table 5**), suggesting that our linear score offers an additional independent predictor. Whilst there
212 was an over-representation of low IPI (0-2) cases observed in the low-risk group of the REMoDL-B
213 cohort, this was not significant (Fisher's exact test $p=0.08$, **Figure 2D**) and, while case numbers are
214 few, neither did we observe a significant enrichment in the 6 existing genetic subgroups defined by
215 Lacy et al ¹³ in the HMRN cohort (Lacy subtype available for 63% of samples, Fisher's exact test
216 $p=0.422$, **Figure 2E**).

217

218 Previous studies have identified a large number of verifiable random gene signatures, associated
219 with outcome in other cancer types ^{31,32} so for completeness, we next compared the prognostic
220 ability of our signature against 300,000 random 30 gene panels in the REMoDL-B and LLMPP

221 datasets, where it outperformed 95.5% of random signatures in the REMoDL-B Data, 98.32% in the
222 LLMPD dataset, and 99.92% in both datasets concurrently (**Supplemental Figure 3**).

223

224 **Signature predicts superior response to ibrutinib in younger DLBCL patients**

225 We were also intent on testing whether our discriminator could identify populations of ABC patients
226 most likely to respond to COO specific therapies. Wilson and colleagues have recently reported
227 superior outcomes of patients in specific subtypes of DLBCL³³. We reasoned that our signature may
228 hold relevance for agents postulated to specifically target ABC-subtype DLBCL. The phase III PHOENIX
229 study examined the addition of ibrutinib to R-CHOP in non-GCB DLBCL. Although ibrutinib addition
230 failed to show benefit across the whole intention to treat (ITT) cohort, in younger patients (<60
231 years), outcomes were indeed superior in the ibrutinib-R-CHOP (I-R-CHOP) arm, with results for older
232 (>60 years) patients seemingly confounded by increased toxicity of the drug. In view of the efficacy in
233 this discrete group of patients, we assessed whether our linear predictor was able to discriminate
234 patients in the PHOENIX cohort with a variable response to ibrutinib, focussing our attention on
235 cases younger than 60 years that were confirmed as ABC sub-type using the HTG EdgeSeq COO Assay
236 (n = 133).

237

238 Altogether patients with high linear predictor scores demonstrated poorer PFS compared to patients
239 classified as low-risk in all patients <60 years of age irrespective of treatment (**Figure 3A**, Low-Risk =
240 57, High-Risk = 76, HR = 2.52, 95% CI = 1.23-5.16, log-rank $p = 0.009$), although OS was only
241 marginally different (**Supplemental Figure 4A**, HR = 1.46, 95% CI = 0.54-3.95, $p = 0.452$). We next
242 considered whether the linear predictor behaved differently in I-R-CHOP and R-CHOP treated
243 patients. For ibrutinib-treated patients (n = 55), both PFS and OS were lower in the high versus low-
244 risk group (**Figure 3B & Supplemental Figure 4B** Low-Risk = 26, High-Risk = 29, HR = 11.6, 95% CI =
245 1.48-90.9, $p = 0.003$ and $p = 0.076$ respectively). Indeed, the low-risk group (47%), had strikingly
246 favourable outcomes, with no deaths reported in these 26 patients and only one patient

247 experiencing progression. It is important to note that the control, R-CHOP arm, demonstrated only a
248 trend to inferior outcomes in the high-risk group in PFS, compared to the significant survival
249 differences observed in the LLMPP, REMoDL-B and HMRN datasets (**Figure 3C & Supplemental Figure**
250 **4C**; Low-Risk = 32, High-Risk = 46, PFS: HR = 1.6, 95% CI = 0.727-3.52, $p = 0.239$ and OS: HR = 1.28,
251 95% CI = 0.429-3.82, $p = 0.656$ respectively).

252

253 Finally, we assessed the effect of ibrutinib addition in high and low-risk linear predictor groups
254 separately. Low-risk patients treated with I-R-CHOP had superior PFS and OS than those treated with
255 R-CHOP only (**Figure 3D**, Ibrutinib = 24, Placebo=33, $p = 0.007$ for PFS; **Supplemental Figure 4D**, $p =$
256 0.028 for OS); while in contrast, the high-risk group showed no difference observed between the
257 treatment arms for either PFS (**Figure 3E**, Ibrutinib = 31, Placebo = 45, HR = 0.927, 95% CI = 0.44-
258 1.95, $p = 0.841$) or OS (**Supplemental Figure 4E**, HR = 0.589, 95% CI = 0.156-2.22, $p = 0.428$). Similar
259 results were shown when examining the non-GCB group of patients. Together, these retrospective
260 data suggest that our gene signature may identify a group of DLBCL patients <60 years who derive
261 benefit from ibrutinib in combination with R-CHOP therapy.

262 Discussion

263 DLBCL comprises a molecularly heterogeneous group of lymphomas with different outcomes, linked
264 to a variety of features including COO²⁵, occurrence of specific translocations³⁴ and more recently a
265 combination of gene mutation and copy number aberrations^{10–13,35}. There are several recently
266 reported discriminators that rely primarily on gene expression, with an emphasis either towards the
267 tumour B cell^{3,36,37}, or its immune microenvironment^{38–41}. However, despite an increased
268 understanding of the biology of these aggressive lymphomas, improvements to the existing standard
269 of care have proven problematic. Altogether, there has been a reliance on the study of the diagnostic
270 biopsy samples, with longitudinal studies typically hindered by the limited availability of sequential
271 biopsy material. Studies comparing mutation status at diagnosis and relapse in paired biopsies, or
272 interrogating independent series of pre-treatment and relapse cases^{15–22} (**Supplemental Table 1**)
273 have identified recurrent relapse-associated genes including *TP53* and *MYC*, although alone lack
274 specificity to predict relapse. In this study, we focussed attention on the changes in gene expression
275 profile that accompany DLBCL relapse, to consider whether this approach might offer a novel
276 perspective on the biology of disease resistance. Our new data demonstrate that COO is largely
277 stable between time points, suggest a distinctive pattern of relapse in ABC and GCB lymphomas
278 based on differential gene expression, and resolve a 30-gene discriminator in ABC-DLBCL that
279 defined clinically distinct low- and high-risk subgroups at diagnosis, that was informative both in an
280 independent series of R-CHOP-treated patients, and young patients treated with ibrutinib + R-CHOP
281 in the PHOENIX trial⁴².

282
283 The accrual of paired material of suitable quality for analysis was challenging. From a large initial
284 series of FFPE paired biopsies obtained from multiple UK institutions, suitably paired data was
285 retrieved from 38 *de novo* DLBCL patients, constituting one of the largest published cohorts of
286 paired diagnosis-relapse samples to date. Regardless, it is important to acknowledge the
287 heterogeneity of the cohort; site of the biopsy differs between the diagnosis and relapse in 18 of the

288 38 pairs; the time to relapse varied across the series and samples demonstrated variable tumour
289 content. Irrespective of these potential confounding effects, we have been able to make some
290 robust observations shedding new light onto the evolution of DLBCL. We had initially sought to
291 recover both DNA and RNA from these specimens, to facilitate a parallel analysis of mutation and
292 gene expression, but this proved technically unfeasible in the majority of cases, highlighting the
293 challenges in collating paired material of sufficient quantity and quality for multi-omic analyses. Our
294 subsequent studies focused exclusively on generating gene expression data, through global GEP and
295 a COO analysis. Comparison of paired biopsies confirmed what has long been assumed, but not
296 formally shown, that COO is stable in most paired diagnostic/relapse cases, ruling out a simple switch
297 in COO as the dominant mechanism underlying disease relapse and R-CHOP failure. Indeed, while
298 changes in COO accompanying DLBCL relapse were observed in 5 cases, this included just a single
299 example of ABC-GCB switching, where biopsies were excised from different locations 1.5 years apart
300 (**Table 1**). While this example is reminiscent of a recent study demonstrating spatial and temporal
301 heterogeneity in a case of DLBCL manifesting as site-discordant COO and response to immuno-
302 chemotherapy⁴³, these data confirm that such discordant cases represent the exception rather than
303 the rule.

304

305 We noted minimal overlap in DE genes between COO groups, with GSEA suggesting that relapse is
306 likely mediated by different mechanisms depending on the tumour's COO. Tumour growth and
307 proliferation signatures were enriched in ABC relapses, while adaptive immunity-related signatures
308 were a feature of GCB type lymphomas. Consequently, we considered ABC (n=17) and GCB (n=11)
309 lymphomas separately for subsequent analysis. We next tested whether these relapse associated
310 genes held prognostic significance in a diagnostic cohort. Using the PAM algorithm we resolved a 30-
311 gene signature that divided ABC cases into low- and high-risk groups. Critically, this expression
312 signature was validated using a linear score in 3 independent GEP datasets derived using different
313 platforms and comprising both population-based and clinical trial cohorts.

314

315 Going forward, it will be important to prospectively validate individual signatures, as well as
316 benchmark them against each other, to determine their relative merits and application in real world
317 patients. While it is reassuring to note in three recent mutation-focussed studies^{10,12,13,35} the
318 significant overlap and consensus across classifications based on gene mutation, it remains to be
319 seen whether the various emerging gene expression-based signatures similarly resolve identical
320 groups of DLBCLs, or rather each identify distinct high-risk groups. Moreover, combined mutation
321 and gene expression data from the HMRN dataset demonstrated that high and low risk patients
322 from our ABC discriminator arose independently of the groups reported by Lacy et al¹³. In contrast,
323 there was limited overlap of patients classified in the Phoenix trial using the LymphGen to allow a
324 direct comparison with our high-low risk patient groups. This data suggests that gene expression
325 profiling imparts important information independent of mutation and CNA-based classifications.

326

327 There is a recognition that genetic signatures, rather than informing clinical decisions based on
328 outcome prediction, may offer instead a tool to identify discrete populations of patients who may
329 benefit from specific precision-based approaches to treatment. It was of interest in our study, that
330 our ABC-discriminator resolved patients with particularly favourable outcome following ibrutinib + R-
331 CHOP in the PHOENIX study within ABC-subtype patients diagnosed at <60 years, albeit in a small
332 retrospective cohort. Importantly, however, in this cohort the discriminator was unable to identify
333 groups with different outcomes in the R-CHOP arm. Ideally, this observation will undergo
334 prospective validation in patients on the upcoming combination study of the BTK inhibitor
335 acalabrutinib with R-CHOP for untreated DLBCL (REMoDL-A:
336 clinicaltrials.gov/ct2/show/NCT04546620) as part of the UK PMAL programme.

337

338 There are certain limitations in our study. Overall, the cohort sizes are small, particularly in the
339 example of GCB-GCB relapse pairs, which may explain the inability to generate a prognostic

340 discriminator for this group of patients. Furthermore, while we employed a biologically agnostic
341 approach to our discriminator discovery, so as not to overlook the impact of unappreciated gene
342 interactions or biology, the resulting discriminator by its nature lacks an immediately apparent
343 biological rationale. However, an interaction network revealed 7 biologically distinct clusters of
344 protein interactions containing several enriched pathways with potential relevance to disease
345 progression, including RNA transport, protein processing and immune pathways. The notable
346 presence of *MYC* at the centre of the interaction network highlights the role of *MYC* in disease
347 aggressiveness and reinforces the need to develop *MYC*-directed therapies.

348

349 The future utility of the many emerging genetic discriminators requires independent validation as
350 part of prospective clinical trials and highlight the need for comprehensive and multi-omic profiling
351 of these cohorts. There are currently limitations in performing direct comparisons between existing
352 GEP studies, e.g. the use of different discovery platforms, and it is possible that fluctuations in the
353 proportion of specific subgroups observed may reflect the unpredictable nature of real-world studies
354 (HMRN) compared with clinical trials (REMoDL-B). Indeed, the inclusion of patients for analysis in
355 many biological studies, are typically dependent on a confirmed lymphoma diagnosis, their
356 treatment, and having sufficient residual material for molecular analysis. In addition, whilst various
357 candidates are being investigated to augment the efficacy of R-CHOP, the performance of the
358 proposed predictive signatures will require re-appraisal in the context of any new standard of care.

359

360 In summary, we have leveraged one of the largest cohorts of paired diagnosis-relapse series in DLBCL
361 demonstrating the stability of COO and derived a 30-gene signature that robustly distinguished low-
362 and high-risk subgroups of ABC patients. This signature also identifies patients who derive benefit
363 from BTK inhibition in combination with R-CHOP adding to the existing toolkit of putative genetic
364 predictors now available in DLBCL that can be readily assessed as part of prospective clinical trials.

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374 **Authorship Contributions:**

375 F.B.C., K.K., S.A., J.G., J.O., P.J., J.W. & J.F. conceived and designed the study.

376 F.B.C., J.W. & J.F. wrote the manuscript.

377 K.K., S.A., E.K., A.C., T.C., M.A.K., S.B., S.V.H., C.B., M.E., S.R., N.C., G.M., M.B., A.N., A.D.,

378 A.S., K.N.N & M.C. collected samples and clinical information.

379 F.B.C, J.W. & J.F. devised methods for analysis.

380 F.B.C, D.J.H. & J.W. performed the bioinformatic analysis.

381 K.K., S.A., D.W.S, L.M.R, performed experiments.

382 C.S., D.W., D.P., B.H., D.J.H, S.B. & A.S. provided access to other data sets.

383 All authors read, critically reviewed, and approved the manuscript.

384 **Competing Interests:**

385 J.F. has provided consultancy and received funding from Epizyme

386 K.K. is an employee and shareholder of Roche

387 D.W.S, L.M.R have IP rights to the Lymph2Cx assay

388 D.W.S. has provided consultancy to Abbvie, AstraZeneca, Celgene, Janssen and Incyte and
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502 observational cohort. *Eur. J. Heal. Econ*. 2017;18(2):255–267.

503

504

505 **Figure Legends**

506 **Figure 1: Gene expression profiles of paired diagnosis and relapse DLBCL biopsies.**

507 38 patients who underwent relapse were included in the study, the clinical features of these patients
508 are shown in **A**. COO remained stable in the majority of cases (**B**). Gene expression profiling was
509 carried out using Ion AmpliSeq™ Transcriptome Human Gene Expression Kit. Principal Component
510 Analysis carried out on these samples suggested poor separation based on timepoint, with a greater
511 degree of separation observed in COO (**C**). Diagnosis = green, relapse = red, ABC = blue, GCB =
512 orange, unclassified = grey, NA=black. Differential gene expression was carried out separately for the
513 stable ABC and GCB cohorts and gene set enrichment analysis was performed, with the number of
514 genes sets dysregulated (FDR <= 0.1) at relapse are shown in **D**. Heatmaps of normalised enrichment
515 score for examples of the dysregulated gene sets are shown in **E**. A 30 gene panel capable of
516 stratifying ABC-DLBCL patients from a training cohort (Reddy et al 2017)¹⁴ into two risk groups with
517 different overall survival was discovered using PAMR (**F**). Red = High Risk, Blue = Low Risk, ** $p <=$
518 0.01, * $p <= 0.05$, . $p <= 0.1$

519

520 **Figure 2: Validation of 30 gene risk model for ABC-DLBCL in population and clinical trial cohorts.**

521 The risk model was tested with survival restricted to 3 years. The risk model, based on the 30 gene
522 panel, was also able to separate data from the REMoDL-B clinical trial⁷ (**A**), the R-CHOP arm of the
523 LLMP cohort (2008)²⁸ (**B**), and the HMRN population study⁴⁴ (**C**). Red = High Risk, Blue = Low Risk. **D**)
524 Comparison of IPI scores and the risk groups defined using the linear predictor in the REMoDL-B
525 cohort. **E**) Comparison of genetic sub-categories described by Lacy¹³ with risk groups defined using
526 the linear predictor in the HMRN cohort. Of the 156 ABC cases in the HMRN data, the genomic sub-
527 groups were available for 98.

528

529 **Figure 3: Prognostic ability of the linear predictor in the PHOENIX trial cohort.** The GEP data from

530 the ABC patients <60 years old in the PHOENIX trial were used to generate linear scores for each

531 patient. These scores were then used to stratify the patients into high and low risk cohorts. Kaplan
532 Meier plots of the PFS of these patient subgroups is shown. **(A)** Both treatment arms combined, only
533 patients designated ABC by GEP. The PFS of these subgroups was also examined in each arm
534 separately, **(B)** Ibrutinib and **(C)** placebo. Red = High Risk, Blue = Low Risk. Finally, the effect of the
535 drugs on PFS within the subgroups was assessed, **(D)** low risk and **(E)** high risk. Green = R-CHOP +
536 placebo, Purple = R-CHOP + Ibrutinib.
537

Figure 1

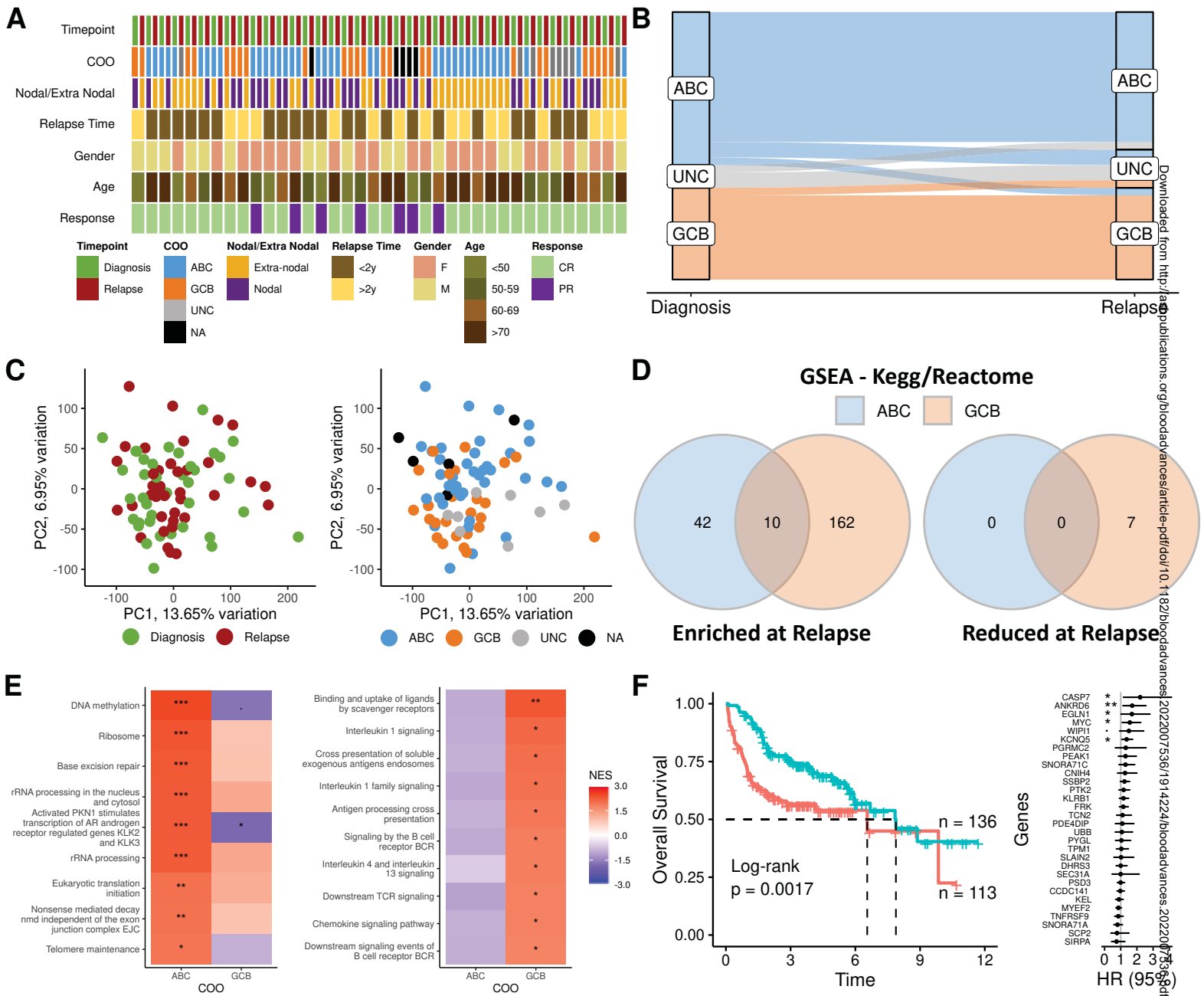


Figure 2

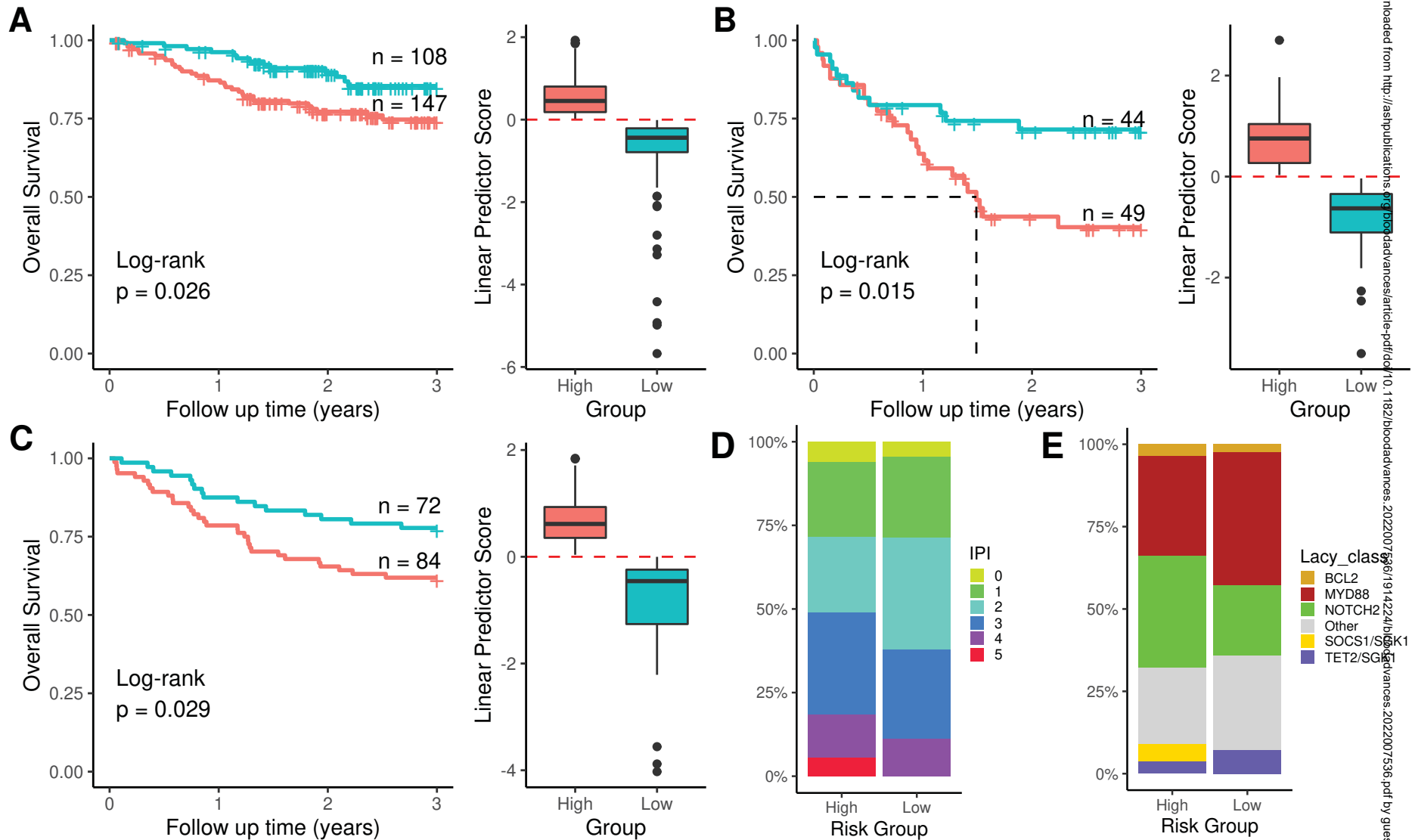


Figure 3

