**Gene expression profiling in an open-label randomised phase 3 trial (REMoDL-B) of bortezomib added to standard chemoimmunotherapy for diffuse large B-cell lymphoma.**

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**Summary**

**Background**: Biologically distinct sub-types of diffuse large B-cell lymphoma (DLBCL) can be identified using gene expression analysis to determine their cell of origin (COO), corresponding to germinal centre (GCB) or activated B‐cells (ABC). This study investigated whether adding bortezomib(B) to standard therapy could improve outcomes in these subtypes.

**Methods**: The REMoDL-B trial is an open-label adaptive two-stage randomised controlled trial at 107 centres in the United Kingdom and Switzerland. Eligible patients (pts) had previously untreated, histologically confirmed DLBCL with sufficient diagnostic material for gene expression profiling and pathology review; age 18 years or older; Eastern Cooperative Oncology Group performance status of ≤2; bulky stage I or stage II-IV requiring full course chemotherapy; measurable disease, and cardiac, lung, renal and liver function sufficient to tolerate chemotherapy. Pts initially received 1 cycle of standard R‐CHOP. During this time, gene expression profiling by whole genome cDNA-mediated annealing, selection, extension and ligation assay was performed on routine diagnostic biopsy material. Patients were then centrally assigned (1:1) via a web-based system, with block randomisation stratified by international prognostic index and COO subtype to continue R‐CHOP +/‐ bortezomib (1.3 mg/m2 IV or 1.6 mg/m2 SC) days 1+8 for cycles 2‐6. The primary endpoint was 30 month progression‐free survival (PFS) for the GCB + ABC population. The primary analysis was intention-to-treat. The safety population consisted of all participants who received at least one dose of study drug. The study was registered at ClinicalTrials.gov: NCT01324596. We report the PFS and safety outcomes for patients in the follow-up phase after the required number of events occurred. Recruitment and treatment has completed for all participants, with long-term follow-up continuing

**Findings**: Between June 2011 and June 2015, 1128 eligible pts were registered and a total of 918 randomised. There was no evidence for a difference in PFS in the combined GCB + ABC population between R‐CHOP (N=361)and RB‐CHOP (N=358) (30 month PFS: 70.6% vs 75.2% respectively) adjusted HR = 0.82, 95% CI 0.63 - 1.08; P=0.16. The most common Grade ≥3 adverse event experienced was haematological toxicity, with 178 (39.8%) and 187 (42.1%) of pts receiving R-CHOP and RB-CHOP experienced, respectively. However, RB‐CHOP was not associated with increased haematological toxicity and 87% of pts completed 6 cycles; Grade ≥3 neuropathy occurred in 3.8% RB‐CHOP *vs* 1.8% R‐CHOP pts. Serious adverse events occurred in 190 (42.5%) and 223 (50.2%) of pts, including 5 and 4 treatment-related deaths in pts receiving R-CHOP and RB-CHOP, respectively.

**Interpretation**: This is the first large-scale study in DLBCL to use real-time molecular characterisation for prospective stratification and randomisation, and subsequent analysis of biologically distinct subgroups. The addition of bortezomib did not improve outcomes in the ABC subgroup as expected, but proteosome inhibition could be investigated as a possible means to improve the treatment of cytogenetic 'double-hit' DLBCL.

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**Introduction**

The combination of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP ) has been considered standard of care for diffuse large B-cell lymphoma (DLBCL) for more than 15 years. Reported 5 year progression free (PFS) and overall survival (OS) in trials of R-CHOP are 70-75% and 75-80% respectively1, although unselected population-based series show lower figures2. Patients with lymphoma that fails to respond or recurs have a poor outlook, with only one third alive at 2 years3. A variety of approaches have been attempted to improve outcomes for these patients, but none has so far increased the OS. The recognised molecular heterogeneity of this aggressive lymphoma contributes to the complexity of this problem.

Gene expression profiling (GEP) of DLBCL has been used to define subgroups with distinct pathogenesis. The cell of origin (COO) classification recognises those cases with gene expression similar to peripheral blood B cells undergoing in-vitro antigen activation, referred to as the activated B-cell (ABC) subtype, whilst the germinal centre B-cell (GCB) subtype resemble B cells in the germinal centre. Retrospective studies suggested that the ABC subtype had worse outcomes, with 3 year progression free survival (PFS) after R-CHOP of the ABC group 40%, compared to 75% in the GCB group4.

The subtypes have distinct genomic characteristics. The ABC subtype shows a higher prevalence of mutations in genes involved in B-cell receptor (BCR) signalling and regulators of NF-κB (*MYD88,CD79B,TNAIP3, CARD11, TRAF2, TRAF5, MAP3K7, TNFRSF11A*), compared with the GCB subtype. Constitutive NF-κB activation downstream of the BCR is a feature of ABC DLBCL. Genomic, pharmacological and RNA interference screens have shown selective oncogenic addiction of the ABC subtype to activation of this protein complex5. Bortezomib is a proteasome inhibitor and may suppress NF-κB activity by preventing proteosomal degration of the inhibitor IκBα, retaining NF-κB as inactive, unable to translocate to the nucleus to mediate transcription. Preliminary clinical studies suggested that bortezomib showed selective efficacy in DLBCL subtypes. When combined with infusional chemotherapy, bortezomib appeared to have preferential activity in relapsed/refractory non-GCB DLBCL specifically with a higher response rate and median OS6.

The Randomised Evaluation of Molecular guided therapy for Diffuse Large B-cell Lymphoma with Bortezomib (REMoDL-B) study (NCT01324596) aimed to investigate the clinical efficacy of R-CHOP in addition to bortezomib in patients with DLBCL. In order to determine whether the COO subtypes respond differently to the combination of bortezomib with R-CHOP we used a study design incorporating prospective randomisation stratified by whole transcriptome gene expression profile. We also incorporated molecular characterisation to examine recognised subgroups distinct from cell of origin; double-hit (DHL) (rearrangements of *MYC* and *BCL-2* and/or *BCL-6*) and double-expressor lymphomas (high MYC and BCL-2 expression) (DEL).

As clinical studies move towards increased application of targeted agents against molecular features, the feasibility of determining a molecular phenotype in real-time was an important objective of the study.

**Methods:**

**Study design and Participants**

The REMoDL-B trial is a multicentre, open-label, randomised, phase 3, superiority trial comparing R-CHOP with R-CHOP plus bortezomib (RB-CHOP) in patients newly diagnosed with DLBCL. As a collaboration between the United Kingdom National Cancer Research Institute (NCRI) group and the Schweiz Arbeitsgemeinschaft für Klinische Krebsforschung (SAKK), patients were recruited from 107 cancer centres in the UK and Switzerland. The trial is registered with ClinicalTrials.gov, NCT01324596. The full study protocol is available in the on-line appendix (pp15-90).Patients were eligible if they had *de novo* DLBCL confirmed by an expert haematopathologist with sufficient diagnostic material for gene expression profiling and central pathology review; age 18 years or older; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or less; bulky stage I or stage II to IV requiring full course chemotherapy; measurable disease, and cardiac, lung, renal and liver function sufficient to tolerate chemotherapy. Patients with a previous history of indolent lymphoma were excluded, but patients with previously undiagnosed concurrent low grade infiltration in bone marrow or lymph node were eligible. Patients with primary mediastinal lymphoma; clinical central nervous system (CNS) involvement; positive serology for HIV, hepatitis B or hepatitis C virus; active malignancy in the preceding 5 years, or other conditions precluding administration of study treatment were ineligible. Pregnant women were also excluded.

At each site the institutional review board approved the protocol. Independent trial oversight was maintained by a trial steering committee (TSC) and the data monitoring and ethics committee (DMEC). The study was carried out according to the principles of the Declaration of Helsinki, Principles of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Good Clinical Practice and in accordance with UK and Swiss regulatory requirements. Written informed consent was obtained from all patients.

**Randomisation and masking**

Participants were centrally randomly assigned (1:1) using block randomisation with varying block size via the web-based TENALEA system7, to receive either R-CHOP or RB-CHOP. Randomisation stratification factors included COO subtype and international prognostic index (IPI). For the purposes of stratification IPI was grouped as: low (0 to 1); intermediate (2 to 3) and high (4 to 5) and those with an unclassified COO subtype were included. In the case of failed RNA extraction/insufficient yield, patients were not randomised but received conventional R-CHOP and followed, with assessments, according to the control arm of the trial. Both participants and treating clinicians were aware of the treatment allocation, however local Investigators were not informed of the results of molecular phenotyping.

**Procedures**

Participants underwent routine staging investigations including computed tomographic (CT) scans and bone marrow biopsy, with examination of cerebrospinal fluid (CSF) examination as clinically indicated. Tumour material was sent to the central laboratory for gene expression profiling (GEP) and somatic mutation assessment.

The R-CHOP regimen (day 1: rituximab 375mg/m2 IV, cyclophosphamide 750mg/ m2 IV, doxorubicin 50mg/ m2 IV, vincristine 1.4mg/m2 (max 2mg) IV, days 1-5 prednisolone 100mg od po) was administered to all patients on a 21 day schedule for cycle 1. From cycle 2 onwards patients were randomised to receive 5 further cycles of R-CHOP in the control arm (Arm A) or 5 cycles of R-CHOP in addition to bortezomib (RB-CHOP) (Arm B) on days 1 and 8 (1.3mg/m2 intravenous (IV) or 1.6mg/m2 subcutaneous (SC)) in the experimental arm. Further cycles were given when neutrophils and platelets had recovered to 1.0 x 109/l and 100 x 109/l respectively, and dose reductions of bortezomib in response to neurotoxicity were closely specified according to the severity (appendix pp48-53). Administration of bortezomib was changed following data supporting reduced toxicity and similar efficacy and greater acceptability to patients with SC administration8. Allopurinol, granulocyte colony stimulating factor (GCSF) and anti-emetic therapy were given according to local policy. Intrathecal prophylaxis with methotrexate was recommended for patients at high risk of CNS relapse for 3 – 6 cycles at investigators’ discretion. Radiotherapy to initial bulk disease, extra-nodal sites or residual masses was according to routine practice in the participating centres. Cross sectional imaging was repeated one month after administration of the final dose of chemotherapy to assess disease response using the International Working Group Response Criteria for Non-Hodgkin Lymphoma, and repeated at 12 months9.

Participants were assessed clinically at each treatment cycle and following treatment completion, every 3 months for a year and thereafter 6 monthly until 5 years’ total follow up. At each assessment, medical history including adverse events, physical examination, ECOG performance status and routine laboratory tests were performed. Progressions were recorded following clinical assessment and imaging, determined by local investigators, according to the standard criteria9; at progression trial treatment was discontinued and patients were followed for survival.

Histological haematoxylin and eosin (H&E) sections from formalin-fixed paraffin embedded (FFPE) samples were reviewed in the central laboratory as a quality check. Macrodissection of tumour was performed by scraping the area of interest from unstained sections on plain microscope slides. RNA was extracted using the Ambion RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (ThermoFisher, Waltham, USA), using the manufacturers protocol, with the exception that 2 washes in xylene and alcohol were used to remove wax, with extended digestion in Proteinase K overnight.

Gene expression profiling was performed using Illumina whole genome cDNA-mediated annealing, selection, extension and ligation (WG-DASL; Illumina, San Diego, USA) assay. Patient samples were classified as either ABC, GCB, Unclassified or Fail using the DASL automated classifier (DAC) previously described, using a quality control of >1 to define technical failure10. The confidence of each sample being one of 3 classes was recorded and the final class defined as that with the highest confidence score.

Where possible, biopsy material was used for construction of tissue micorarrays (TMA) for immunohistochemistry, Fluorescent In-Situ Hybridisation (FISH), and DNA extraction. Specifically, immunohistochemistry for MYC and BCL-2 (dual) expression was performed on TMA (n=358) using Abcam (Cambridge, UK; clone Y69) and Dako anti-BCL-2 (Agilent, Santa Clara, USA; clone 124) monoclonal antibodies, scored by two independent assessors according to recognised criteria using cutoffs of ≥40% and ≥50% respectively. In the event of a disagreement of score, a third observer would arbitrate. In cases where it was not possible to define whether a sample was positive or negative, these were defined as borderline. Using these criteria, cases with high or average MYC and high or average BCL-2 expression were used to define cut-off values for correlated mRNA levels. These were used to identify *MYC* and *BCL-2* gene expression categories: high or average.

DNA was extracted from tumour cells enriched by microdissection on FFPE tissue sections and its quality assessed by PCR of variously-sized genomic fragments. A panel of 70 genes that are recurrently mutated in aggressive B-cell lymphomas were investigated for mutation by targeted sequencing using HaloPlexHS target enrichment and Illumina HiSeq sequencing as described previously11. This was carried out in a total of 395 cases where DNA was available with adequate quantity and quality. Duplicate experiments were performed in 61 cases, including all those with quality control PCR showing amplification of ≤300bp genomic fragments, and only those mutations which were reproducible in both samples were reported. Samples with better quality were investigated in a single replicate. Variant calling, SNPs and background noise filtering were as previously described11. In a further 22 cases, mutations in 20 genes (included in the above 70 gene panel) were analyzed in duplicate using Fluidigm multiplex PCR and Illumina MiSeq sequencing as described previously11.

Variants detected by the above targeted sequencing methods were further assessed by functional prediction tools and those predicted to be benign by ≥7 out of 9 programs, not in the COSMIC database, were excluded. The resulting variants were further scrutinized by reviewing the BAM file to eliminate any potential PCR/sequence artefacts. As part of a post hoc analysis, samples were tested for the possible presence of primary mediastinal lymphoma using a Bayesian predictor described by the Lymphoma Molecular Profiling Project12.

**Outcomes**

The primary outcome was PFS, defined as time from registration to the date of progression or death from any cause. Disease progression was determined using the International Working Group Response Criteria for Non-Hodgkin Lymphoma14. Participants free from progression or death were censored at the date of their last visit.Secondary outcomes were the time-to-event variables of overall survival (OS), event-free survival; disease-free survival and time to progression; response duration; complete and overall response rates; evaluation of toxicity; quality of life and assessment of peripheral neuropathy.

**Statistical analysis**

An adaptive design was used based on a two-stage approach, with two interim analyses to explore respectively the safety and efficacy in the GCB group treated with RB-CHOP after defined numbers of events. The first interim analysis was to take place once 55 patients in the GCB group receiving RB-CHOP patients were randomised. If the one year PFS was assessed to be below 70% in the subgroup, the trial would stop recruiting in the GCB group. The second interim analysis was to take place when 73 patients in the GCB group receiving RB-CHOP patients were randomised and followed for one year. If the one year PFS was assessed to be below 85% in the subgroup, the trial would stop recruiting in the GCB group.

The trial was powered to detect an improvement in 30 month progression-free survival (PFS) of 10% in the combined ABC+GCB groups, from 75% in the R-CHOP arm to 85% in the RB-CHOP arm (corresponding to a hazard ratio of 0.56) , based on a log-rank test, with a significance level of 5% (two-sided) and 90% power, requiring a total of 129 events. The Intention-to-treat (ITT) population was formed of all patients for whom GEP was attempted (ABC, GCB, Unclassifed or GEP failed). The safety population was formed of all patients in the ITT population who received at least one dose of any study drug.

The primary outcome of PFS (time-to-event) was assessed using a Cox proportional hazards model, adjusted for COO subtype and IPI score, carried out in the ABC+GCB ITT population.

Secondary outcome analyses included repeating the primary outcome analysis in the ABC ITT population, the GCB ITT population and the Unclassified ITT population; adjusting for IPI score only. Kaplan-Meier curves were produced for time-to-event data and follow-up maturity was described using reverse Kaplan-Meier method. Summary statistics were used to describe baseline characteristics for R-CHOP, RB-CHOP and GEP Fail patients in the ITT population, and for COO subtypes for the ITT population; with formal comparisons between COO subtypes carried out using Pearson Chi-squared tests. Toxicity information was summarised by treatment arm, and post-hoc analyses included comparing toxicity information by treatment using Pearson Chi-squared tests for the Safety Population. These also included repeating the primary outcome analysis, adjusting for time from diagnosis to the start of treatment to ascertain whether diagnosis to treatment interval impacted PFS outcome. Additional post-hoc analyses also included assessing PFS and OS by treatment arm in the combined ABC + GCB populations for IPI Low, Intermediate and High groups respectively, and repeating the primary outcome analysis but excluding patients who had a dose reduction in any treatment drug. There was no adjustment for multiple comparisons and Stata statistical software (version 15.1) was used for all analyses.

**Role of the funding source**

The trial was funded by an educational grant from Janssen-Cilag and endorsed by CRUK following independent peer review (C328/A12128). Bortezomib was supplied free of charge by Janssen-Cilag. Translational work was supported by a Bloodwise specialist programme grant to the Precision Medicine for Aggressive Lymphoma consortium (15002). The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**Results**

Between 2nd June 2011 and 10th June 2015, 1128 participants were registered to the study.  Fifty-two (4.6%) of 1128 did not meet the eligibility criteria, of whom 29 were deemed ineligible following tumour biopsy, 5 had insufficient tumour data, 5 were withdrawn by clinician choice, 2 by patient choice, 1 patient died before reaching the point of randomisation and 10 withdrew for other reasons not specified. This resulted in 1076 (95.4% of 1128) patients reaching the point of attempted GEP randomisation (ITT population).  One-hundred and fifty-eight patients (14.7% of 1076) had inadequate material for GEP and subsequent randomisation ('Fail', received R-CHOP), whilst 918 (85.3% of 1076) were stratified by COO subtype and IPI for random assignment to receive R-CHOP or RB-CHOP (Figure 1). Following the planned interim analyses and safety assessments by the DMEC in the GCB group, the trial continued to recruit to all groups. In all, 35 (3.3%) of 1076 patients did not receive any study drug and 35 (3.3% of 1076) were lost to follow up during the study period.

Baseline characteristics were similar between the control arm, experimental arm and non-randomised participants (Table 1).  Median turnaround time from the tumour material reaching the diagnostic lab to a COO result being available was 8 days (interquartile range 6-12). The overall failure rate was 14.6%, mainly due to insufficient tissue remaining in the block.  In those cases where sufficient material was extracted, only 1% failed for technical reasons

There were clinical differences between the molecular sub-groups (Table 2). Median age was higher in the ABC type (67 versus 63, p=0.005) and there was more often bulky disease in the GCB type 158 of 475 (33.8%) GCB; 50 of 244 (20.7%) ABC; and 55 of 119 (27.8%) Unclassified: p<0.0001). Patients with bone marrow involvement were over-represented in the unclassified type: 66 of 475 (14.2%) GCB, 33 of 244 (13.8%) ABC and 42 of 199 (22%) Unclassified, p=0.017). There was however no significant difference in the distribution of IPI risk groups, elevated serum LDH, conventional stage, overall prevalence of extranodal disease or ECOG performance status between the different molecular subtypes (appendix table 1, p6). Testing as described for the possible presence of primary mediastinal lymphoma, a total of 19 cases fulfilled the criteria, of which 13 had mediastinal disease. Of these, 14 had been allocated to the GCB group and 5 unclassified.

The panel of genomic mutations confirmed the known association of different somatic changes with COO subtypes, with a bias towards alterations in epigenetic modifier genes in the GCB group and genes of the B-cell receptor signalling pathway in the ABC group. Thus, mutations in *EZH2* were seen in 23% of GCB but only 3.4% of ABC biopsies, and conversely mutations in *MYD88* were found in 7% of GCB and 44.4% of ABC samples tested (Appendix Figure 1, p1 and Appendix table 2, pp6-7). NF-κB target genes were preferentially expressed in the ABC group compared with the GCB group (Appendix figure 2, p2).

The addition of bortezomib to R-CHOP was well tolerated (Table 3). The most common grade ≥3 adverse event experienced was haematological toxicity, in 178 (39.8%) and 187 (42.1%) of pts receiving R-CHOP and RB-CHOP, respectively. However, there was no significant increase in rates of grade ≥3 neutropenia, febrile neutropenia, thrombocytopenia or anaemia (p>0.05). Neuropathy of any grade was more frequent in RB-CHOP treated patients (p<0.0001) (Appendix Table 3, pp8-11) but there was no significant difference in the event rate for neuropathy of grade ≥3. Serious adverse events were experienced in 190 (42.5%) of 447 R-CHOP pts vs 223 (50.2%) of 444 RB-CHOP pts. Nine Suspected Unexpected Serious Adverse Reactions (SUSARs) were reported: four in the R-CHOP arm (haemophagocytic syndrome, leukaemia secondary to chemotherapy, neutropenic sepsis, and fracture); and five in the RB-CHOP arm (jejunal stricture with small bowel obstruction, bowel perforation, renal failure, sepsis and tumour lysis syndrome). There were fewer dose reductions overall in the R-CHOP arm than the RB-CHOP arm: 158 (34.5%) of 459 vs 196 (42.9%) of 459, respectively. There were also fewer discontinuation from trial treatments observed in the R-CHOP arm compared to the RB-CHOP arm: 43 (9.4%) of 459 vs 60 (13.1%) of 459, respectively (Appendix Table 4, p11). However, median relative dose intensity (RDI) for patients in the control and experimental arm was similar for the agents of R-CHOP and a high proportion of patients successfully completed 6 cycles of treatment: 418 (91.3%) of 459 and 398 (87.1%) of 459, respectively(Appendix Table 5, p12). The number of deaths experienced in the R-CHOP arm and the RB-CHOP arm in the safety population were: 73 (16.3%) of 447 vs 68 (15.3%) of 444, respectively, with the majority of deaths due to progressive lymphoma (R-CHOP: 50 [68.5%] of 73; RB-CHOP: 54 [79.4%] of 68), and 9 treatment related deaths reported (R-CHOP: 5 [6.8%] of 73; RB-CHOP: 4 [5.9%] of 68) (Appendix Table 6, p13).

The primary outcome efficacy analysis was performed when median follow-up of the combined ABC+GCB ITT population reached 30 months, as stipulated in the protocol (median [95% CI]: 29.7 [29.0 to 32.0]; median follow-up of survivors [95% CI]: 29.4 [28.6 to 31.1]). The number of PFS events (progression/deaths) observed in this group was 198 (107 in R-CHOP and 91 in RB-CHOP), and the number of OS events (deaths) observed was 116 (62 in R-CHOP and 54 in RB-CHOP). There was no difference in PFS in the combined ABC + GCB populations between the R-CHOP and RB-CHOP groups; adjusted HR = 0.84, 95% CI 0.64 - 1.11; P=0.23

Although no adjustment for multiple testing was made, efficacy analyses were repeated after additional follow-up was received: median follow-up of survivors in the ABC+GCB ITT population [95% CI]: 42.3 months [40.9 to 45.6]. The number of PFS events (progression/deaths) observed in this group was 211 events (115 in R-CHOP and 96 in RB-CHOP), and the number of OS events (deaths) observed was 133 (72 in R-CHOP and 61 in RB-CHOP). There was no evidence of difference in PFS in the combined ABC + GCB populations between the R-CHOP and RB-CHOP groups; adjusted HR = 0.82, 95% CI 0.63 - 1.08; P=0.16 (Figure 2A) . Estimated 30 month PFS rates (PFS30) were 70.6% (95% CI: 65.5% to 75.0%)and 75.2% (95% CI: 70.3% to 79.4%) for R-CHOP and RB-CHOP respectively. Analysis of subtypes by COO showed that bortezomib did not significantly affect PFS in either ABC (adjusted HR = 0.78, 95% CI 0.51-1.21; *P* = 0.27) (Figure 2B), GCB (adjusted HR 0.85, 95% CI 0.60-1.20; P=0.35) (Figure 2C), or Unclassifiable cases (adjusted HR 1.29, 95% CI 0.77-2.16; P=0.34) (Figure 2D). There was no difference in OS by arm in the ITT ABC and GCB population (n=719); adjusted HR 0.82, 95% CI 0.59-1.16; P=0.27). Estimated OS at 30 months was 81.6% (95% CI: 77.1% to 85.3%) vs 83.1% (95% CI: 78.7% to 86.7%) (Appendix Figure 3, p3). Post-hoc analyses were undertaken, assessing the PFS and OS by treatment arm in the combined ABC + GCB populations for IPI Low, Intermediate and High groups respectively (Appendix Figure 4, p4). These showed a statistically significant improvement in PFS for RB-CHOP patients with Low IPI score (adjusted HR= 0.43, 95%CI 0.21 to 0.91; P=0.026), but no other statistically significant differences in PFS between R-CHOP and RB-CHOP. There were no statistically significant differences between R-CHOP and RB-CHOP groups for OS in any of the IPI subgroups. The median time from diagnosis to first treatment was similar between arms (R-CHOP 17 days [Q1, Q3: 10, 29]; RB-CHOP 20 days [Q1, Q3: 10, 32]. Post-hoc analyses, adjusting for the time from diagnosis to first treatment interval also showed no difference in PFS in the combined ABC + GCB populations between the R-CHOP and RB-CHOP groups; adjusted HR = 0.83, 95% CI 0.56 - 1.24; P=0.36. Similarly, post-hoc analyses excluding patients who had a dose reduction in any treatment drug showed no difference in PFS in the combined ABC + GCB populations between the R-CHOP and RB-CHOP groups; adjusted HR = 0.80, 95% CI 0.54 - 1.19; P=0.27.

We analysed recognised prognostic subgroups within the trial population (Appendix Table 7, p13). Karyotypic double-hit lymphomas (DHL) were rare in the ABC population and were significantly associated with the GCB subtype (0.4% vs 6.7%, p<0.0001). Conversely, the ABC subtype was associated with high concomitant expression of MYC and BCL-2 proteins compared with the GCB subtype (excluding DHL) (54.9% vs 26%, p<0.0001) and mRNA (44.7% vs 18.3%, p<0.0001) (Appendix Table 7, p13).

Among the patients receiving R-CHOP, *MYC*-rearrangement, DHL and dual high *MYC* and *BCL-2* mRNA expression were significantly associated with inferior PFS after controlling for IPI. Cases with either DHL (N=35) or dual high expression of *MYC* and *BCL-2* mRNA (n=207) had significantly worse PFS at 30 months (48.6% vs 76.0%, adjusted HR [95% CI]: 2.32 [1.43 to 3.78], p=0.00070; and 65.1% vs 76.0%, adjusted HR [95% CI]: 1.56 [1.20 to 2.04], p=0.00096, respectively)(Figure 3). High MYC and BCL-2 by immunohistochemistry did not show significant effects upon outcomes, although the number of cases was limited, resulting in wide confidence limits.

In these high-risk subgroups, for DHL the HR in patients who received RB-CHOP compared with R-CHOP was 0.53, 95% CI 0.2 - 1.37; P=0.19; for concomitant high MYC and BCL-2 mRNA expression HR = 0.70, 95% CI 0.45 - 1.09; P=0.11, and for double expression MYC and BCL-2 by immunohistochemistry HR = 0.6 95% CI 0.29-1.51 P=0.16 (Figure 4).

We examined the effect of the addition of bortezomib on survival in patients bearing mutations known to be associated with activation of NF-κB, the putative target of bortezomib (*MYD88, CD79A, CD79B, TNFAIP3, TNFRSF11A*) and found no significant differences for single gene alterations (Appendix Figure 5, p5).

**Discussion**

This trial has demonstrated the feasibility of molecular phenotyping in a large multi-centre study of a rapidly-progressive tumour and shown that the addition of bortezomib does not influence treatment outcomes in most cases of DLBCL. Patients entering clinical trials are often not representative of the wider population, and for prospective testing of a complex biomarker we wished to avoid worsening the problem of generalisability by restricted enrolment and delays to the initiation of therapy. This was done by studying routine FFPE biopsy material and deferring randomisation to cycle 2 of R-CHOP, allowing treatment to start as soon as staging investigations were completed, with molecular analysis taking place in parallel. There is a further advantage to deferred introduction of an experimental agent; 62% of treatment related deaths have been reported following the first cycle in DLBCL out of all cycles delivered13, this might be worsened by an additional drug.

All previous studies of GEP in lymphoma have been performed retrospectively, but assignment of patients to novel therapies based upon their molecular phenotype will require real-time outputs, which we have shown to be feasible in this trial. Using a central laboratory and the DAC classifier we could prospectively assign COO categories in a clinically relevant timeframe. This allowed a stratified randomisation, with the potential for adaptive design based on interim analyses of molecular subtypes. The accuracy of the classifier is supported by finding the expected frequencies of different mutations that are known to be enriched in ABC or GCB respectively (Figure 2 and Appendix Table 2, pp6-7)14. We identified NF-κB target genes to be more highly expressed in the ABC subtype whilst almost all cases of DHL were identified within the GCB population, consistent with the literature (Appendix Table 7, p13)15. Dual expression of MYC and BCL-2 proteins or RNA was more frequent in ABC cases and at similar frequencies to that previously reported (Appendix Table 7, p13).

The overall frequency of the ABC subtype (27%) was lower than has been reported in some retrospective series, where roughly equal numbers of GCB and ABC cases were seen15,16. However, a recent large randomised trial reported very similar findings to the present study, with 58% GCB, 26% ABC and 16% unclassified cases, using the NanoString Lymphoma Subtyping Test17. It is possible that some cases of lymphoma with poor prognostic features at presentation are excluded from trials such as this on the grounds of performance status or the need for urgent treatment before screening procedures can be completed, and this may reduce the proportion of ABC type cases entering prospective studies.

The overall outcomes are consistent with other large prospective studies in DLBCL, with a similar PFS (72% at 30 months) to recent phase III trials1,17. The PFS for the ITT population was not improved by the addition of bortezomib at these doses, nor was there evidence of a differential effect according to the COO. This is in keeping with the findings of a smaller randomised phase II study where the addition of bortezomib 1.3 mg/m2 to R-CHOP on days 1 and 4 did not improve outcomes for non-GCB DLBCL, defined in that study by immunohistochemistry18.

Delivery of the R-CHOP was not significantly compromised by the addition of bortezomib. Individual R-CHOP components had similar mean received dose intensity between arms and almost 90% of patients completed 6 cycles. Although more dose reductions were noted in the RB-CHOP group, and this might have countered a positive effect of the addition of bortezomib, the difference was small and consisted mostly of reductions in vincristine dose. The slight increase in neurotoxicity suggests that the bortezomib was given at a biologically active dose. It was administered on days 1 to 8 of cycles 2 to 5, at a dose which has shown efficacy in other lymphoma trials, but we recognise that more potent proteasome inhibitors are now in use, as are other agents with apparent preferential effects in the ABC group such as lenalidomide and ibrutinib, trials of which are in progress.

Bortezomib did not improve outcomes in the ABC subtype, which was confirmed to be enriched for expression of NF-κB target genes, or for cases with somatic mutations associated with NF-κB activation. It is possible that inhibition of NF-κB is insufficient to improve outcomes in addition to R-CHOP in DLBCL, or that bortezomib at the doses used was unable to inhibit NF-κB adequately for this to occur. A different study in patients with non-GCB lymphoma, selected by immunohistochemistry, failed to demonstrate a difference between R-CHOP and R-CHP, with bortezomib given in place of vincristine, supporting this finding19.

Recent studies have demonstrated the difficulty of improving the results of initial therapy in DLBCL by the addition of novel agents which had shown promising activity in single arm studies treating recurrent disease. This may partly reflect biological selection through treatment failure: in the relapsed/refractory lymphomas where new agents are investigated, biology is likely to be different from the newly-diagnosed. Thus, the majority of GCB DLBCL are cured by R-CHOP, while recurrent disease is enriched for those with DHL. Similarly recurrent ABC DLBCL is enriched for double-expressor lymphomas, which may account for the different results reported from the previous series of bortezomib treatment. This highlights the need for full molecular characterisation of the diseases being treated, both at diagnosis and in the case of initial treatment failure.

The presence of a small number of DHL within the GCB group has lowered the PFS figure for this subgroup in this study. Overall however, the outcome for the DHL cases appears better than has been reported in some earlier studies20 and consistent with more recent analyses21,22. Whilst clearly worse than the non-rearranged group, it appeared that nearly half of DHLs had not progressed at 30 months. The PFS at 30 months was 38.9% after R-CHOP compared with 58.9% after RB-CHOP, although this was the result of a post-hoc analysis and was not statistically significant (data not shown).

A number of limitations can be identified in this study. Any clinical trial is potentially prone to selective recruitment of better-prognosis patients, but we endeavoured to minimise this effect by deferring randomisation until the second cycle of therapy, allowing rapid initiation of treatment with molecular typing proceeding in tandem. As a result, the median time from diagnosis to initiation of therapy was lower than in comparable studies23, and the distribution of IPI scores in this study was similar to, or worse, than recent trials1,17, with 47% cases high-intermediate or high risk. Despite this, the exclusion of patients with performance status of 3 or above may have removed a cohort with the most adverse biology. It was not possible to perform central histopathology review on the cases entered and the presence of Epstein-Barr virus (EBV) in the biopsy material was not routinely examined, but all were diagnosed by expert haematopathologists, a procedure with a high accuracy for DLBCL: over 96% in a recent series from the UK24, and EBV is present in less than 3% of DLBCL in Europe, making it unlikely that this would have affected the results. The dose of bortezomib was chosen to reflect that used in other combination studies but could be regarded as lower than optimum, owing to the risk of additive neurotoxicity. There was also a slight excess of vincristine dose reductions in the RB-CHOP arm, which could potentially have eroded a positive effect from the bortezomib. The use of routine FFPE biopsy material was necessitated by the large number of recruiting centers, but resulted in a failure rate of 15% for molecular typing, and may have resulted in a larger than expected number of unclassified cases where poor quality RNA resulted in a low probability score in the COO classifier.

In conclusion, this trial has demonstrated that complex molecular characterisation can be performed in real time, with a pragmatic treatment schedule that allows for the allocation of therapy based upon the molecular sub-type. This is likely to become increasingly relevant as our understanding of the phenotypic diversity of DLBCL expands to encompass not only COO, but other biologically distinct categories based upon genomic alterations as well15,25,26. Future trials using such methods will be important to explore the mechanisms of action of investigational agents and to re-define the groups in which they are most likely to be effective, with the poor prognosis double hit lymphomas representing a potential opportunity for such an approach.

**Research in context panel:**

**Evidence before this study.**

We searched PubMed from January 1998 until March 2014 for publications in English for reported randomised clinical trials with the terms “diffuse large B-cell lymphoma” and “cell of origin”, and studies involving “diffuse-large B-cell lymphoma” and “bortezomib”.

Using gene expression profiling to characterise patients, ABC subtype had inferior survival compared with the GCB subtype in several retrospective patient cohorts treated with R-CHOP or CHOP-like regimens. Bortezomib had limited single-agent activity in DLBCL but had been successfully combined with standard chemotherapy regimens. Bortezomib in combination with dose-adjusted R-EPOCH (rituximab, etoposide, cyclophosphamide, doxorubicin, vincristine, and prednisolone) resulted in higher progression free survival in the ABC than the GCB subtype. Bortezomib in combination with R-CHOP produced similar outcomes in non-GCB DLBCL (ascertained by immunohistochemistry) compared with GCB DLBCL.

**Added value of the study.**

To our knowledge, the study is the first to combine prospective gene expression profiling of lymphoma with a targeted therapy to allow stratification and randomisation within a phase 3 clinical trial. It is the first study to assess a novel agent in DLBCL prospectively powered to address subtypes defined by gene expression profiling, and demonstrates that the addition of bortezomib to R-CHOP does not improve survival as expected in the ABC subgroup. Extensive characterisation and subgroup analyses suggest that COO subtype and NF-κB activating mutations do not associate with improved outcomes with RB-CHOP, and that bortezomib does not act as an effective inhibitor of the NF-κB pathway in this disease. Exploratory analyses however suggest that different high-risk subgroups, DEL and DHL, may benefit from the addition of bortezomib or similar agents to standard immunochemotherapy.

**Implications of all the available evidence.**

The trial design provides a rational framework for future studies in DLBCL, allowing prompt initiation of treatment while molecular characterisation is carried out. The study confirms R-CHOP as the standard of care for most patients with DLBCL, but raises the possibility that high-risk subgroups could benefit from the addition of a proteasome inhibitor to standard therapy, which may guide future research.

**Data sharing statement**

Individual participant data will be made available, including data dictionaries, for approved data sharing requests.  Individual participant data will be shared that underlie the results reported in this article, after de-identification and normalisation of information (text, tables, figures, and appendices). The study protocol is available in the appendix (appendix pp15-90) and statistical analysis plan will also be available upon request .  Anonymised data will be available beginning 3 months after and ending 5 years following publication of the article, to researchers who provide a completed Data Sharing Agreement that describes a methodologically sound proposal, for the purpose of the approved proposal. Proposals should be directed to ctu@soton.ac.uk. Data will be shared once all relevant parties approve and sign the Data Sharing Agreement. Data sharing requests are available for 5 years via the Southampton CTU website.

**Contributors**

PJ and AD designed the study, had oversight and contributed to data interpretation, writing and approval of the report. TC and GG contributed to data analysis, interpretation, writing of the report and oversaw work at the Southampton Clinical Trials Unit. SB undertook the molecular, histological and cytogenetic analysis, and contributed to writing of the report. TM and LS contributed to designing the study, central statistical data monitoring, the writing and development of the statistical analysis plan and writing of the report. TM also carried out the main clinical statiscal analyses and production of the correspoinding figures and tables of the report. CS, DW, MC and RT performed bioinformatic analysis and data interpretation. DW, RT and MC also contributed to design, implementation and testing of the COO classifier. CB and RT performed histological analysis and approved the report. FC, AC and MD performed mutational analysis, data interpretation and contributed to writing of the report. JC managed the trial and contributed to writing of the report. SKS was involved in project management and approval of the report. RS contributed to trial design and oversight. AM, PF, GC and CP gathered and interpreted the data. All authors have reviewed and approved the final version of the report.

**Declaration of interests**

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Figure 1: Consort diagram outlining randomisation



**Figure 2:** PFS according to treatment arm A: ITT population: GCB + ABC patients N=719 (Labels indicate the estimated percentage progression-free at 12 months and 30 months) B: ABC subgroup(N=244) C: GCB subgroup (N=475) D: Unclassified group. (N=199)

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**Figure 3a.** Progression-free survival comparing double-hit lymphomas to non-rearranged cases, separated by treatment arm

**Figure 3b** Progression-free survival comparing double-expressor (High *MYC* and high *BCL-2* mRNA) lymphomas to all other cases, separated by treatment arm

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**Figure 4.** Forest plot of hazard ratios based on PFS for high-risk and different molecular subtypes by treatment arm.

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristic** | **R-CHOP** **(N=459)** | **RB-CHOP** **(N=459)** | **Non-rand.** **(n=158)** |
| **Median age (range)** | **65 (24-86)** | **63 (20-84)** | **65 (24-85)** |
| **ECOG Performance Status – n (%)** |  |  |  |
| **0** | **250 (56.4%)** | **222 (50.1%)** | **73 (47.4%)** |
| **1** | **142 (32.1%)** | **168 (37.9%)** | **57 (37.0%)** |
| **2** | **51 (11.5%)** | **53 (12.0%)** | **24 (15.6%)** |
| **Bone marrow involved - n (%)** |  |  |  |
| **Yes** | **78 (17.4%)** | **63 (14.1%)** | **34 (21.7%)** |
| **Serum LDH Level – n (%)** |  |  |  |
| **>ULN** | **224 (59.4%)** | **227 (61.7%)** | **77 (71.3%)** |
| **IPI score – n (%)** |  |  |  |
| **Low (0-1)** | **123 (26.8%)** | **120 (26.1%)** | **33 (20.9%)** |
| **Low Intermediate (2)** | **11 (24.2%)** | **123 (26.8%)** | **45 (28.5%)** |
| **High Intermediate (3)** | **145 (31.6%)** | **133 (29.0%)** | **51 (32.3%)** |
| **High (4-5)** | **80 (17.4%)** | **83 (18.1%)** | **29 (18.4%)** |
| **Stage – n (%)** |  |  |  |
| **I** | **12 (2.6%)** | **14 (3.1%)** | **5 (3.2%)** |
| **II** | **131 (28.7%)** | **126 (27.6%)** | **37 (23.6%)** |
| **III** | **128 (28.0%)** | **154 (33.7%)** | **48 (30.6%)** |
| **IV** | **186 (40.7%)** | **163 (35.7%)** | **67 (42.7%)** |
| **Bulk greater than 10cm - n (%)** |  |  |  |
| **Yes** | **122 (26.8%)** | **141 (31.3%)** | **66 (42.6%)** |
| **Molecular phenotype: ABC% xd**  | **121 (26.4%)** | **123 (26.8%)** | - |
| **Molecular phenotype: GCB%**  | **240 (52.3%)** | **235 (51.2%)** | - |
| **Molecular phenotype: Unc%**  | **98 (21.4%)** | **101 (22.0%)** | - |

Table 1. Baseline characteristics of the participants by treatment arm (ITT population and non-randomised cases). ECOG= European Cooperative Oncology Group (Unc= unclassified)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Characteristic** | **ABC****(n=244)** | **GCB****(n=475)** | **Unc****(n=199)** | ***P* value** **ABC vs GCB** |
| **Age Median (range), years** | **67 (22-86)** | **63 (20-82)** | **63 (20-84)** | **0.0045** |
| **ECOG Performance Status – n (%)** |
| **0** | **121 (51.9%)** | **247 (53.8%)** | **104 (53.6%)** |  |
| **1** | **84 (36.1%)** | **158 (34.4%)** | **68 (35.1%)** | **0.83** |
| **2** | **28 (12.0%)** | **54 (11.8%)** | **22 (11.3%)** |  |
| **Bone Marrow Involved – n (%)** | **33 (13.8%)** | **66 (14.2%)** | **42 (22.0%)** | **0.017** |
| **Serum LDH Level >ULN – n (%)** | **115 (60.8%)** | **231 (59.8%)** | **105 (61.8%)** | **0.19** |
| **IPI score – n (%)** |
| **Low** | **66 (27.0%)** | **127 (26.7%)** | **50 (25.1%)** |  |
| **Low Intermediate** | **70 (28.7%)** | **117 (24.6%)** | **47 (23.6%)** | **0.822** |
| **High Intermediate** | **69 (28.3%)** | **144 (30.3%)** | **65 (32.7%)** |  |
| **High** | **39 (16.0%)** | **87 (18.3%)** | **37 (18.6%)** |  |
| **Stage – n (%)** |
| **I** | **8 (3.3%)** | **12 (2.5%)** | **6 (3.0%)** |  |
| **II** | **76 (31.1%)** | **134 (28.5%)** | **47 (23.6%)** | **0.74** |
| **III** | **73 (29.9%)** | **148 (31.4%)** | **61 (30.7%)** |  |
| **IV** | **87 (35.7%)** | **177 (37.6%)** | **85 (42.7%)** |  |
| **Bulk greater than 10cm – n (%)** | **50 (20.7%)** | **158 (33.8%)** | **55 (27.8%)** | **<0.0001** |

Table 2. Clinical characteristics of COO subtypes (Unc= unclassified) (ITT population)

|  |  |  |
| --- | --- | --- |
|  | **R-CHOP (n=447)** | **RB-CHOP (n=444)** |
|  | **Grade 1-2** | **Grade 3** | **Grade 4** | **Grade 5** | **Grade 1-2** | **Grade 3** | **Grade 4** | **Grade 5** |
| Any Adverse event | 414 (92.6%) | 226 (50.6%) | 107 (23.9%) | 6 (1.3%) | 415 (93.5%) | 253 (57.0%) | 105 (23.6%) | 4 (0.9%) |
| Haematological  | 115 (25.7%) | 128 (28.6%) | 96 (21.5%) | 1 (0.2%) | 118 (26.6%) | 153 (34.5%) | 89 (20.0%) | 1 (0.2%) |
| Neutropenia  | 51 (11.4%) | 107 (23.9%) | 92 (20.6%) | 1 (0.2%) | 62 (14.0%) | 137 (30.9%) | 81 (18.2%) | 1 (0.2%) |
| Thrombocytopenia  | 22 (4.9%) | 5 (1.1%) | 2 (0.4%) | 0 | 36 (8.1%) | 7 (1.6%) | 7 (1.6%) | 0 |
| Anaemia | 73 (16.3%) | 19 (4.3%) | 0 | 0 | 82 (18.5%) | 14 (3.2%) | 0 | 0 |
| Neuropathy  | 183 (40.9%) | 8 (1.8%) | 0 | 0 | 249 (56.1%) | 17 (3.8%) | 0 | 0 |
| Nausea or Vomiting  | 160 (35.8%) | 7 (1.6%) | 0 | 0 | 194 (43.7%) | 15 (3.4%) | 1 (0.2%) | 0 |
| Febrile neutropenia | 8 (1.8%) | 49 (11.0%) | 14 (3.1%) | 0 | 7 (1.6%) | 51 (11.5%) | 9 (2.0%) | 0 |
| Neutropenic sepsis | 3 (0.7%) | 9 (2.0%) | 23 (5.1%) | 1 (0.2%) | 3 (0.7%) | 19 (4.3%) | 11 (2.5%) | 1 (0.2%) |
| Febrile Neutropenia or Neutropenic sepsis  | 11 (2.5%) | 55 (12.3%) | 33 (7.4%) | 1 (0.2%) | 10 (2.3%) | 67 (15.1%) | 20 (4.5%) | 1 (0.2%) |
| Abdominal pain | 61 (13.6%) | 12 (2.7%) | 1 (0.2%) | 0 | 64 (14.4%) | 9 (2.0%) | 0 | 0 |
| Alopecia | 114 (25.5%) | 9 (2.0%) | 0 | 0 | 106 (23.9%) | 6 (1.4%) | 0 | 0 |
| Constipation | 165 (36.9%) | 1 (0.2%) | 0 | 0 | 180 (40.5%) | 5 (1.1%) | 0 | 0 |
| Cough | 53 (11.9%) | 0 | 0 | 0 | 63 (14.2%) | 1 (0.2%) | 0 | 0 |
| Diarrhoea | 95 (21.3%) | 10 (2.2%) | 0 | 0 | 133 (30.0%) | 24 (5.4%) | 0 | 0 |
| Dyspnoea | 56 (12.5%) | 4 (0.9%) | 0 | 0 | 59 (13.3%) | 4 (0.9%) | 0 | 0 |
| Fatigue | 201 (45.0%) | 10 (2.2%) | 0 | 0 | 191 (43.0%) | 8 (1.8%) | 0 | 0 |
| Fever | 64 (14.3%) | 17 (3.8%) | 1 (0.2%) | 0 | 87 (19.6%) | 14 (3.2%) | 1 (0.2%) | 0 |
| Mucositis | 73 (16.3%) | 2 (0.4%) | 0 | 0 | 62 (14.0%) | 6 (1.4%) | 0 | 0 |
| Nausea | 141 (31.5%) | 3 (0.7%) | 0 | 0 | 165 (37.2%) | 8 (1.8%) | 1 (0.2%) | 0 |
| Pain | 56 (12.5%) | 5 (1.1%) | 0 | 0 | 69 (15.5%) | 6 (1.4%) | 0 | 0 |
| Peripheral sensory neuropathy | 129 (28.9%) | 3 (0.7%) | 0 | 0 | 182 (41.0%) | 8 (1.8%) | 0 | 0 |
| Sepsis | 2 (0.4%) | 2 (0.4%) | 12 (2.7%) | 0 | 0 | 2 (0.5%) | 15 (3.4%) | 0 |
| Vomiting | 63 (14.1%) | 6 (1.3%) | 0 | 0 | 109 (24.5%) | 11 (2.5%) | 0 | 0 |

**Table 3: A**dverse events (safety population). Adverse events for which grade 1 or 2 events were reported in 10% or more of patients, adverse events for which grade 3, 4, or 5 events were reported in 2% or more of patients, and any other haematological and neutropenia related adverse events reported.