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HIV-associated DLBCL: Clinicopathological factors including dual-colour chromogenic in situ hybridisation to assess *MYC* gene copies

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ABSTRACT

Introduction: Diffuse large B-cell lymphoma (DLBCL) comprises up to 43% of non-Hodgkin lymphomas in South Africa due to the high seroprevalence of human immunodeficiency virus (HIV) infection in the southern African region. We explored the prognostic influence of an array of clinicopathological factors, including *MYC* gene copy numbers, within HIV-associated DLBCL.
Methods: The retrospective inclusion of 123 tumours was followed by c-MYC immunohistochemistry and dual-colour *MYC* and centromere 8 (CEN8) chromogenic in situ hybridisation on formalin-fixed paraffin-embedded sections. Clinicopathological data were collected, interpreted and analysed.
Results: HIV seropositive patients comprised 81% (93/115), mean age 42 (SD 10.8) years, with 55% males, HIV negative patients comprised 19% (22/115), mean age 57 (SD 16.7) years ($p = 0.001$), with 59% males and the HIV status was unknown for 8 patients. The median CD4 count was 162 (IQR 215) cells/mm^3, 33% of patients presented with CD4 counts <100 cells/mm^3 and the median viral load was 217 (IQR 182 981) copies/mL. There was advanced stage at presentation (i.e., III-IV, 87%), with Ki-67 proliferation indices ≥90% in 85%- and c-MYC expression (i.e., ≥40%) in 58% of tumours. Double expression of c-MYC and BCL2 was associated with a non-germinal center immunophenotype ($p < 0.01$). Low-level increase of *MYC* gene copy numbers and *MYC* rearrangements occurred in 57% and 12%, respectively. C8 polysomy, *MYC* gene clusters and concurrent *MYC* rearrangement/increased *MYC* gene copies were also detected. Inferior median overall survival (OS) occurred when the CD4 counts were <100 cells/mm^3 (149 days 95% CI 44-254, p 0,04) and when IPI scores were 3–5 [155 days (95% CI 37–273), $p = 0.01$]. Concomitant infections negatively impacted the survival outcome, multivariate regression analysis (HR 4.01, 95% CI 1.86–12.20, $p = 0.02$).
Conclusion: c-MYC protein expression, low-level increase in *MYC* gene copy numbers, rearrangement, C8 polysomy, *MYC* gene clusters and concurrent *MYC* rearrangement/low-level gains are present in HIV+ DLBCL. CD4 counts < 100 cells/mm^3, IPI scores 3-5 and concomitant infections negatively impact the survival outcome.

1. Introduction

Up to 30% of cancers in Africa are linked to infectious agents and in the context of the aggressive B-cell non-Hodgkin lymphomas (NHL), human immunodeficiency virus (HIV) infection is an established risk factor [1,2]. Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 35% of malignancies of lymphoid origin [3]. Notably, within developing countries such as South Africa (SA), DLBCL comprises 43% of NHL due to the high seroprevalence of HIV infection in the southern African region [4,5]. Four to 16% of de novo DLBCL may harbour *MYC* translocations as detected by fluorescence in situ hybridisation [FISH] [6-8]. Moreover, FISH, dual-colour chromogenic in situ hybridisation (CISH) and comparative genomic hybridisation techniques have been utilised to demonstrate increased *MYC* gene copies in 3-83% of DLBCL [8-13]. Increased *MYC* copies in DLBCL is associated with c-MYC protein overexpression and the latter correlates with a poor overall survival [11]. *MYC* copy number aberrations in DLBCL confers an inferior median overall survival (OS) and an inferior progression free survival [13]. Increased *MYC* gene copy numbers in HIV-associated DLBCL have yet to be explored in depth. Therefore, in a context of

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high HIV seroprevalence, we aimed to identify increased *MYC* gene copy numbers in DLBCL by utilising dual-colour CISH and c-MYC immunohistochemistry (IHC). The prognostic influence of an array of clinicopathological factors was explored thereafter.

2. Methods and materials

A retrospective observational study was conducted on DLBCL in patients at Chris Hani Baragwanath Academic Hospital, Soweto, South Africa. A systematised nomenclature of medicine (SNOMED) search of the laboratory database occurred for histopathologically confirmed DLBCL from October 2013 to June 2017. IHC panels during the diagnostic work up included CD20 (clone L26, Ventana Medical Systems [VMS], Roche Diagnostics, Tucson, AZ, USA), CD10 (clone SP67, VMS), BCL2 (clone 124, VMS), bcl-6 (clone GI191E/A8, VMS), MUM1 (clone SP114/MRQ-43, VMS) and Ki67 (clone 30–9 VMS). The tumour morphology and IHC sections of all the cases were reviewed. IHC was semi quantitatively assessed in 10% increments and assigned an overall tumour proportion positivity of 0–100%. CD20, CD10, bcl-6 and MUM1 IHC were interpreted as positive when ≥30% of tumour cells displayed appropriate immunoreactivity and BCL2 was positive when ≥50% of tumour cells demonstrated cytoplasmic staining. c-MYC IHC (clone Y69, VMS) was performed for this study and nuclear expression in ≥40% of the tumour cells was interpreted as positive.

Dual-colour, *MYC* and chromosome 8 centromere (CEN8), CISH was conducted on formalin-fixed paraffin-embedded (FFPE) tissue sections, 2 μm in thickness, in a BenchMark XT automated instrument (Ventana Medical Systems, Inc., Roche Diagnostics). A *MYC* dinitrophenol (DNP) probe (980–1218), the ultraView silver in situ hybridisation (SISH) DNP detection kit, the red in situ hybridisation (ISH) digoxigenin (DIG) detection kit, chromosome 8 (CEN8) DIG probe (980–1220), accessory reagents and the XT dual-colour open probe staining procedure were utilised. The *MYC* probe hybridises an approximately 496 kilobase region of C8 that includes the *MYC* gene. The *MYC* target sequence is visualised within nuclei by light microscopy as black dots of similar size and intensity to the internal control cells. The CEN8 DIG probe is an analyte specific reagent that was designed to hybridise to CEN8 deoxyribonucleic acid (DNA). Dual colour open probe staining occurred by combining the *MYC* DNP probe with the CEN8 probe, in accordance with manufacturer specifications. Hereby, CEN8 ISH occurred simultaneously and the reaction was visualised with red detection chemistry. A nucleus would display two black signals of *MYC* and two red signals of CEN8. Dual-colour CISH sections were deemed evaluable when *MYC* and CEN8 signals were identified within the nuclei of epithelial cells, lymphocytes, macrophages, fibroblasts or endothelial cells of the external control tissue, i.e. tonsil, and the non-neoplastic internal control cells. Mapped to the area of highest c-MYC immunoexpression within a tumour, 50 tumour cells were selected within the CISH section of each tumour for signal enumeration under light microscopy at x400 magnification. Aligned with previous CISH-based DLBCL studies [9,11], increased *MYC* gene copy numbers and increased CEN8 signals were present when their respective counts were >10% above normal, i.e. >110 signals per 50 tumour nuclei or >2.2 signals per nucleus of tumour. The subcategorisation of DLBCL then occurred as follows:

- Not increased *MYC* gene copy numbers and not increased CEN8 signals i.e. ≤110 *MYC* gene signals and ≤110 CEN8 signals per 50 tumour nuclei.
- Increased *MYC* gene copy numbers, i.e. >110 MYC gene signals and ≤110 CEN8 signals per 50 tumour nuclei.
- Increased *MYC* gene copy numbers i.e. >110 *MYC* gene signals and increased CEN8 signals i.e. >110 CEN8 signals per 50 tumour nuclei.
- *MYC* gene clusters were defined as tight aggregates of signals that were not visually separable.

Age, gender, HIV status and viral load, CD4 count, topographic site of biopsy, morphology, immunophenotype, FISH for *MYC* rearrangement, Ann Abor stage of lymphoma, International Prognostic Index (IPI) score, treatment, concomitant conditions and the survival outcome of patients were documented until December 2021.

2.1. Statistical analysis

IBM SPSS Statistics 28.0 software was used for data analysis. Categorical data were compared using Fisher's exact or Pearson Chi square tests (2-tailed). The Mann-Whitney *U* test or the independent samples *t*-test was used for comparison of medians or means of the non-/normal distributed continuous variables, respectively. Overall survival (OS) was applicable from the date of the DLBCL diagnosis to the date of death, from any cause, or the date last seen alive. Kaplan-Meier method was used to estimate the survival functions and compared by log-rank test. Data were censored. To assess the impact of prognostic factors on OS, Cox proportional hazards model was developed. To deteremine potential associations of prognostic factors with survival outcome, univariate analysis was performed and hazard ratios (HR) were calculated with 95% confidence intervals (CI). Factors with a p value ≤ 0.02 or those which are clinically relevant were selected as the covariates for multivariate analysis. Significance was met if the probability (*P*) value was <0.05 alpha.

3. Results

One hundred and thirty-six cases were retrieved from the laboratory database. After excluding DLBCL transformed from low-grade B-cell NHL, high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements, B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classic Hodgkin lymphoma, Burkitt lymphoma and cases with limited tumour cells, 123 ($N = 123$) DLBCL patients were included. The HIV status was unknown for 8 patients. The HIV negative patients comprised 19% (22/115), the mean age was 57 (SD 16.7) years and 59% were males. There was an 81% (93/115) predominance of HIV seropositive patients, the mean age was 42 (SD 10.8) years and 55% were males. CD4 counts were available for 86 patients. The median CD4 count was 162 (IQR 215) cells/mm^3 and the CD4 count was <100 cells/mm^3 in 33% (28/86) of the patients. HIV viral load was available for 78 patients and the median viral load was 217 (IQR 182 981) copies/mL. The viral load was lower than detectable limit (LDL) for 30% (23 of 78) of the HIV+ DLBCL patients. At the initial presentation of lymphoma, the HIV seropositive patients were significantly younger ($p = 0.001$), demonstrated an advanced stage of lymphoma, i.e., Ann Arbor stage III-IV (48/55, 87%, $p = 0.04$) and the Ki-67 proliferation indices were often ≥90% (78/92, 85%, $p = 0.01$) as shown in Table 1. The HIV status was not significantly associated with other clinicopathological factors such as bone marrow infiltration ($p = 1.00$), IPI scores ($p = 1.00$), concomitant conditions ($p = 0.94$), topographic site of biopsy [nodal or extranodal] ($p = 0.46$), starry-sky (SS) appearance ($p = 0.42$), cytomorphology ($p = 0.28$), cell of origin (COO) immunophenotype (germinal center [GC] or nongerminal center [NGC] ($p = 0.81$) and double expression (DE) of BCL2 and c-MYC ($p = 0.91$).

HIV+ DLBCLs positively expressed CD10, bcl-6, MUM1 and BCL2 in 44%, 85%, 66% and 41%, respectively (Table 1). Co-expression of MUM1 and bcl-6 occurred in 53% of these tumours. The IHC COO was assigned according to the Hans et al. [14] defined algorithm. GC and NGC immunophenotypes were present in 53% and 47% of the HIV+ DLBCLs, respectively. c-MYC IHC was successful on 94% (116 of 123) of the DLBCLs. c-MYC expression (≥40%) was detected in 40% and 58% of the HIV- and HIV+ DLBCLs, respectively. Double expression (DE) of BCL2 and c-MYC proteins occurred in 25% of HIV+ DLBCLs and DE was significantly associated with the NGC COO immunophenotype ($p = 0.002$) as shown in Table 2. HIV+ DLBCL with DE was not significantly associated with age ($p = 0.32$), stage ($p = 1.00$), IPI score ($p = 0.52$), increased *MYC* copies ($p = 0.32$), *MYC* rearrangement ($p = 0.36$) or *MYC*

Table 1
Immunophenotypic analysis of DLBCL.

Immunomarker	DLBCL	HIV+ DLBCL	HIV− DLBCL	P-value
CD20+ (≥30%)	99% (122/123)	99% (92/93)	100% (22/22)	1.00 (Chi-square test)
+proportion (min–max)	80–100%	80–100%	100%	
average proportion+	99%	99%	100%	
CD10+ (≥30%)	45% (54/121)	44% (40/91)	46% (10/22)	0.83 (Chi-square test)
+proportion (min–max)	30–100%	30–100%	40–90%	
average proportion+	69%	68%	63%	
bcl-6+ (≥30%)	82% (98/119)	85% (76/89)	73% (16/22)	0.16 (Chi-square test)
+proportion (min–max)	30–90%	30–90%	40–90%	
average proportion+	69%	67%	76%	
MUM1+ (≥30%)	67% (81/121)	66% (61/92)	71% (15/21)	0.65 (Chi-square test)
+proportion (min–max)	30–90%	30–90%	30–90%	
average proportion+	61%	59%	63%	
BCL2+ (≥50%)	43% (51/118)	41% (36/88)	59% (13/22)	0.13 (Chi-square test)
+proportion (min–max)	50–100%	50–100	60–100%	
average proportion+	75%	73%	82%	
Ki-67				0.01 (Chi-square test)
<90	20% (25/122)	15% (14/92)	41% (9/22)	
≥90 (%)	80% (97/122)	85% (78/92)	59% (13/22)	
min–max	70–≥95%	70–≥95%	70–95%	
average proportion+	89%	90%	86%	
c-MYC+ (≥40%)	55% (64/116)	58% (52/89)	40% (8/20)	0.13 (Chi-square test)
+proportion (min–max)	40–90%	40–80%	40–70%	
average proportion+	56%	56%	54%	

Abbreviations: CD – cluster of differentiation.

aberration ($p = 0.15$). There were no significant differences in the median OS when DE occurred in HIV+ DLBCL (DE+ 320 days, 95% CI 0-783, DE- 257 days, 95% CI 0-681, $p = 0.93$), when DE occurred with NGC COO (DE+ NGC 320 days, 95% CI 54-586, DE+ GC 398 days 95% CI 0-1546, $p = 0.55$) and when DE occurred with rituximab immunotherapy ($p = 0.49$). Although multivariate regression analysis demonstrated a HR of 9.48 (95% CI 0.23 - 10.77) for DE, this did not approach significance ($p = 0.24$).

After c-MYC IHC occurred, dual colour CISH was performed on 51 tumours that contained sufficient viable tumour within tissue blocks. CISH was successful on 46 tumours. *MYC* copies were also assessed in 4 tumours by utilising FISH at the time of initial diagnosis. The HIV status was positive for 42, negative for 7 and unknown for 1 case that underwent *MYC* copy number enumeration. Overall, increased *MYC* copy numbers were detected in 28 tumours (56%, 28/50), inclusive of four cases that were detected using FISH (Table 3 and Fig. 1). Low-level increase in *MYC* gene copy numbers occurred in 24 of the HIV+ DLBCLs (57%, 24/42) and 4 of the HIV- DLBCLs (57%, 4/7) as shown in Table 3. Notably, there were two DLBCLs (HIV+ and -) with C8 polysomy (Fig. 2), two cases of HIV+ DLBCL with *MYC* gene clusters (Fig. 3) and one case of HIV+ DLBCL that concurrently harboured *MYC* rearrangement and increased copies of *MYC*. Bivariate analysis of

Table 2
IHC cell of origin and double expression in DLBCL.

	Overall DLBCL	HIV+ DLBCL	HIV- DLBCL	P-value
IHC cell of origin				0.81 (Fischer's exact test)
GC	65/119 (55%)	48/90 (53%)	12/21 (57%)	
NGC	54/119 (45%)	42/90 (47%)	9/21 (43%)	
Double expression (c-MYC and BCL2)	28/110 (26%)	21/83 (25%)	5/20 (25%)	0.98 (Chi-Square test)
DE & COO				[HIV+] 0.002 (Chi-Square test)
DE+ GC	9/27 (33%)	5/20 (25%)	3/5 (60%)	
DE+ NGC	18/27 (67%)	15/20 (75%)	2/5 (40%)	[HIV-] 1.00 (Fischer's exact test)

Abbreviations: IHC – immunohistochemistry, GC – germinal center, NGC – nongerminal center, DE – double expression.

Table 3
MYC copies increased.

No.	DLBCL study no.	Age (yrs) Gender(M/F) Topography (lymph node -LN/extranodal - EN)	*MYC* signals per 50 nuclei; per nucleus of tumour	CEN8 signals per 50 nuclei; per nucleus of tumour	c-MYC IHC (proportion+)
1.	D1	39/M/LN	116; 2.32	89; 1.78	50%
2.	D4[a]	32/F/EN	128; 2.56	89; 1.78	80%
3.	D5	45/F/LN	113; 2.26	100; 2.00	70%
4.	D8[d]	38/F/EN	114; 2.28	92; 1.84	70%
5.	D12	48/F/EN	122; 2.44	99; 1.98	70%
6.	D18	23/F/LN	116; 2.32	94; 1.88	60%
7.	D19	49/F/EN	137; 2.74	95; 1.90	50%
8.	D20	38/F/EN	116; 2.32	94; 1.88	40%
9.	D22	57/M/LN	116; 2.32	72; 1.44	15%
10.	D29	48/M/LN	111; 2.22	94; 1.88	70%
11.	D33	44/M/LN	126; 2.52	92; 1.84	60%
12.	D40	32/M/EN	135; 2.70	94; 1.88	50%
13.	D41	55/F/EN	112; 2.24	96; 1.92	0%
14.	D42	49/M/EN	122; 2.44	93; 1.86	50%
15.	D50[d]	77/M/LN	111; 2.22	103; 2.06	0%
16.	D52[e]	38/F/EN	Clusters	100; 2.00	80%
17.	D60	31/F/LN	155; 3.10	81; 1.62	40%
18.	D61[b]	55/M/EN	150; 3.00	–	30%
19.	D70[e]	37/M/LN	Clusters	103; 2.06	70%
20.	D71[bd]	32/M/EN	150; 3.00		40%
21.	D85	43/F/EN	113; 2.26	85; 1.70	80%
22.	D86	45/M/EN	117; 2.34	75; 1.50	40%
23.	D87[c]	46//M/LN	132; 2.64	122; 2.44	50%
24.	D89[b]	47/M/LN	150; 3.00	–	70%
25.	D90	61/F//LN	118; 2.36	91; 1.82	40%
26.	D111[b]	17/M/LN	150; 3.00	–	10%
27.	D112[cd]	48/F/EN	144; 2.88	117; 2.34	50%
28.	D115	28/M/LN	181; 3.62	78; 1.56	50%

Note: Numerical assessment of CISH signals was limited by a two-dimensional view of three-dimensional nuclei.

[a] D4 concurrently harboured *MYC* rearrangement.
[b] Increased copies detected using FISH: 4 DLBCLs.
[c] C8 polysomy: 2 DLBCLs.
[d] DLBCL with increased *MYC* gene copies occurred in 4 HIV negative patients.
[e] *MYC* clusters: 2 DLBCLs.

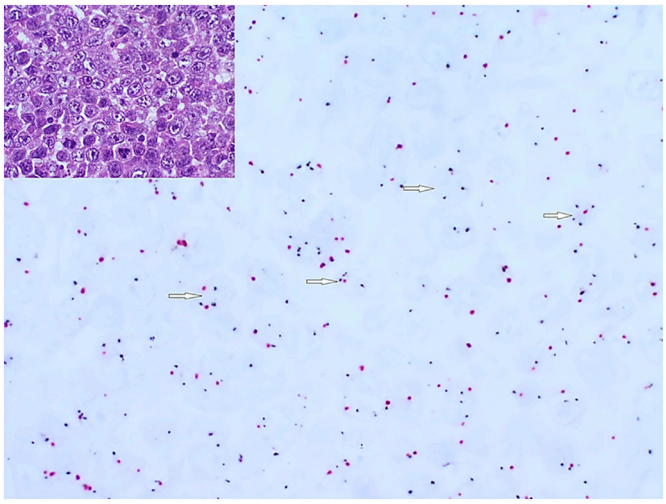


Fig. 1. Increased *MYC* gene copies within HIV+ DLBCL (dual colour CISH, 40× objective).

clinicopathological factors and increased *MYC* gene copy numbers is shown in Table 4.

MYC break-apart or t(8;14) FISH was performed on 33 HIV+ DLBCLs and 6 HIV- DLBCLs at the time of the initial diagnosis. *MYC* rearrangement was detected in 12% (4/33) of HIV+ DLBCLs and was not detected in HIV- DLBCLs. *MYC* aberrations (rearrangement and/or increased *MYC* copies) were present in 47% (27/57) of HIV+ DLBCL and 44% (4/9) of HIV- DLBCLs ($p = 1.00$ Fischer's exact test).

Survival outcome data were available for 76 patients (60 HIV+ and 16 HIV-) who were referred to the Clinical Haematology Unit, Department of Medicine at Chris Hani Baragwanath Academic Hospital (CHBAH). The median follow-up time was 1359 days [3.7 years] (95% CI 692–2026 days). The median OS was 228 days (95% CI 54–402) in the HIV+ group and 825 days (95% CI 309–1341) in the HIV- group ($p = 0.08$, log rank test). The HIV+ DLBCL subgroup with CD4 < 100 cells/mm^3 experienced a significantly inferior median OS (149 days [95% CI 44-254]) compared to the HIV negative group (825 days [95% CI 309-1341]), ($p = 0.04$) as shown in Fig. 4.

A limited number of patients (22 HIV+, 12 HIV-) received rituximab immunotherapy in combination with chemotherapy (cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone). Rituximab immunotherapy significantly improved the median OS in the overall

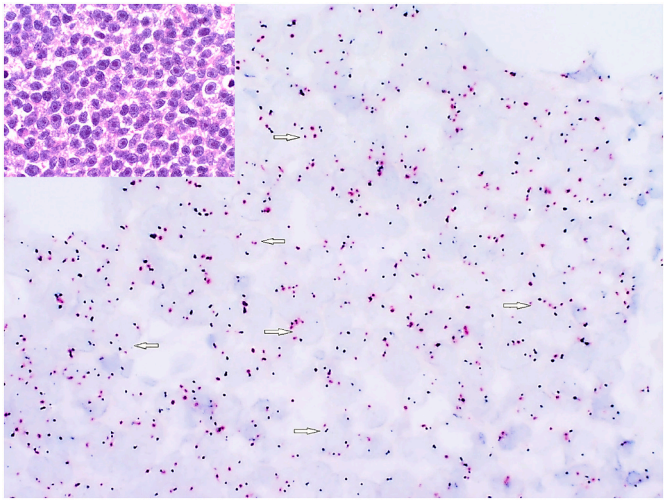


Fig. 2. Increased *MYC* gene copies with C8 polysomy within HIV+ DLBCL (dual colour CISH, 40× objective).

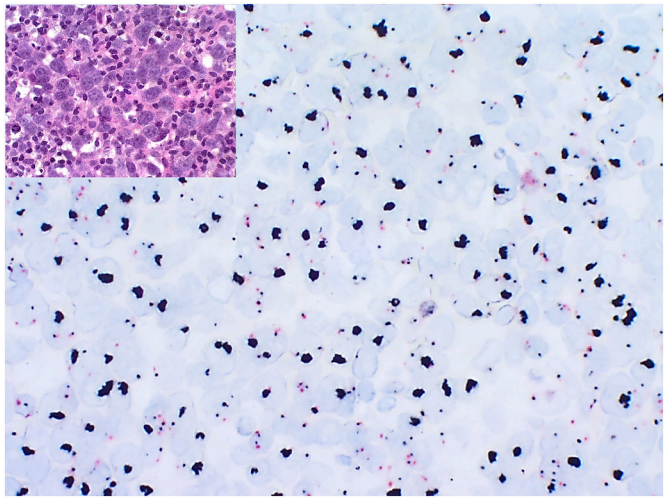


Fig. 3. *MYC* gene clusters (black/silver-stained aggregates) within HIV+ DLBCL (dual colour CISH at 40× objective).

HIV+ DLBCL group from 109 days (95% CI 37–181) to 325 days (95% CI 0–673) [$p = 0.002$, log rank]. Five HIV+ patients with CD4 counts <100 cells/mm^3, received rituximab and the median OS outcome in this limited subgroup was 530 days (95% CI 0–1110). When this median OS was compared with the median OS [825 days (95% CI 384–1266)] of the HIV- DLBCL patients on rituximab a statistically significant difference in the median OS was not demonstrated ($p = 0.63$ log rank). Infection concomitant conditions (CCIs) were documented in 24 HIV+ patients and CCIs were not significantly associated with CD4 counts <100 or ≥100 cells/mm^3 ($p = 0.61$ Chi-square test). The median OS was 257 days (95% CI 0-764) when HIV+ DLBCL patients had CD4 counts <100 cells/mm^3, CCIs and received rituximab ($p = 0.06$, log rank test). Multivariate regression analysis demonstrated proclivity towards a favourable survival outcome (HR 0.04, 95% CI 0.001–1.66, $p = 0.09$) when rituximab immunotherapy was administered. No significant differences in the median OS were demonstrated when HIV+ DLBCLs were substratified as CD4 <150 cells/mm^3 [257 days (95% CI 16–498)] and ≥150 cells/mm^3 [320 days (95% CI 87–553) $p = 0.49$] or CD4 < 200 cells/mm^3 [257 days (95% CI 35–479)] and ≥200 cells/mm^3 [320 days (95% CI 130–510) $p = 0.63$] and compared with the HIV negative group [825 days (95% CI 384–1266), $p = 0.26$ and p = 0.22, respectively].

Inferior median OS was demonstrated in the HIV+ DLBCL group when the IPI scores were 3–5 [155 days (95% CI 37–273), IPI 0-2 228 days (95% CI 0-460) $p = 0.01$] and when CCIs were present [154 days (95% CI 85–223, no CCIs 228 days (95% CI 54-402), $p = 0.01$]. Multivariate regression analysis demonstrated a significant HR of 4.01 (95% CI 1.86–12.20, $p = 0.02$) when CCIs were present. However, the HR when the IPI was 3–5 did not retain significance (HR 4.44, 95% CI 0–5.66 $p = 0.99$). c-MYC protein expression did not signficantly influence the median OS (c-MYC+325 days 95% CI 45–605 and c- MYC- 228 days 95% CI 143–313 [p = 0.86]). The median OS of the GC subtype was 398 days (95% CI 0–1083) and the NGC subtype was 105 days (95% CI 44–166), [p = 0.10] in the HIV+ DLBCLs.

MYC aberrations and DE in DLBCL demonstrated HR >1, as shown in Table 5 which were not statistically significant by multivariate regression analysis.

4. Discussion

HIV-associated (+) DLBCL is an aggressive high-grade malignancy and the survival outcome is influenced by an array of clinicopathological factors.

Table 4
Bivariate analysis of clinicopathological factors and increased MYC gene copy numbers.

Clinicopath factors	Overall DLBCL increased *MYC* gene copies	HIV+ DLBCL increased *MYC* gene copies	HIV− DLBCL increased *MYC* gene copies	*P* value
Increased *MYC* gene copy numbers	n = 28/50 (56%)	n = 24/42 (57%)	n = 4/7 (57%)	1.00 (Fischer's exact test)
Minimum	111	111	111	
Maximum	181	181	150	
Mean (SD)	129 (18)	129 (18)	130 (20)	
Age (median, IQR) yrs	44 years (16)	44 (16)	43 (36)	0.62 Mann Whitney U test
Gender F/M	F 13/28 (46%) M 15/28 (54%)	F 11/24 (46%) M 13/24 (54%)	F 2/4 (50%) M 2/4 (50%)	1.00 (Fischer's exact test)
IPI				1.00 (Fischer's exact test)
0–2	4/18 (22%)	4/16 (25%)	0/2 (0%)	
3–5	14/18 (78%)	12/16 (75%)	2/2 (100%)	
Stage				1.00 (Fischer's exact test)
I–II	1/21 (5%)	1/17 (6%)	0/4 (0%)	
III–IV	20/21 (95%)	16/17 (94%)	4/4 (100%)	
COO				1.00 (Fischer's exact test)
GC	13/27 (48%)	11/23 (48%)	2/4 (50%)	
NGC	14/27 (52%)	12/23 (52%)	2/4 (50%)	
c-MYC				1.00 (Fischer's exact test)
≥40%	23/28 (82%)	20/24 (83%)	3/4 (75%)	
<40%	5/28 (18%)	4/24 (17%)	1/4 (25%)	
DE+	12/28 (43%)	10/24 (42%)	2/4 (50%)	1.0 (Fischer's exact test)
DE−	16/28 (57%)	14/24 (58%)	2/4 (50%)	
Ki-67				1.0 (Fischer's exact test)
<90%	4/28 (14%)	4/24 (17%)	0/4 (0%)	
≥90%	24/28 (86%)	20/24 (83%)	4/4 (100%)	
Median OS				0.50 (Log-rank test)
Increased *MYC* copies	257 days (95% CI 0–540)	105 days (95% CI 0–391)	825 (95% 0–914)	HIV+ p = 0.52
Not increased *MYC* copies	418 days (95% CI 0–914)	752 days (95% CI 0–1635)	134 (95% CI 0–914)	HIV- p = 0.22

Abbreviations: F-female, M-male, COO - cell of origin, GC- germinal center, NGC -nongerminal center, DE - double expression.

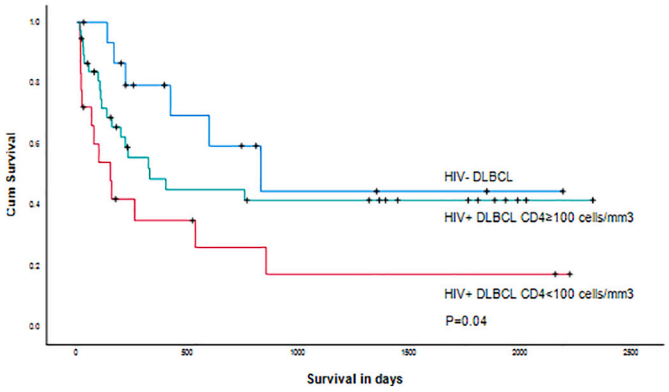


Fig. 4. Kaplan-Meier survival estimates: HIV- DLBCL, HIV+ DLBCL CD4 < 100 and HIV+ DLBCL CD4 ≥ 100 [cells/mm^3]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HIV+ DLBCL tends to occur approximately two decades earlier at 36 to 41 years of age [5,15,16] compared to DLBCL, NOS, which occurs in HIV-negative patients at approximately 59 to 70 years [3,17]. Our findings align herewith as the HIV seropositive patients were significantly younger at presentation of lymphoma [mean age of 42 (SD 10.8) years], than the HIV negative patients [mean age of 57 (SD 16.7) years (p = 0.001]. There was a predominance of male patients in the HIV+ (55%) and the HIV- (59%) groups.

South Africa (SA) currently has the largest population of people who are living with HIV infection [18]. Therefore, in this study there was an 81% (93/115) predominance of HIV seropositivity which mirrors the high seropositivity rate that was documented at our institute in a preceding DLBCL study [15]. In the current study, the patients received combination antiviral therapy (cART) prior to lymphoma presentation or commenced cART soon after the presentation with lymphoma. The median CD4 count, within days to 18 months of the lymphoma diagnosis, was 162 (IQR 215) cells/mm^3 and the median viral load was 217 (IQR 182 981) copies/mL. Thirty three percent (28/86) of the HIV+ DLBCL patients presented with profound immunosuppression with CD4 counts <100 cells/mm^3. When the CD4 count is severely compromised

Table 5
Univariate and multivariate Cox regression analysis of selected clinicopathological factors associated with overall survival.

Clinicopathological factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
HIV+ CD4 < 100 cells/mm^3 and HIV−	3.08	1.16–8.17	0.02	1.96	0.02–2.17	0.78
CC infections	2.93	1.55–5.51	<0.001	4.01	1.86–12.20	0.02
IPI (3–5)	5.72	1.35–24.29	0.02	4.44	0.00–5.66	0.99
Rituximab	0.27	0.13–0.55	<0.001	0.04	0.00–1.66	0.09
DE	1.07	0.53–2.19	0.84	9.48	0.23–10.77	0.24
MYC aberration	0.84	0.40–1.77	0.64	1.23	0.05–5.67	0.89

Abbreviations: HIV – human immunodeficiency virus, + positive, − negative, CC – concomitant conditions, IPI – International Prognostic Index, DE – double expression of c-MYC and BCL2 proteins, HR – hazard ratio.

in HIV seropositive patients, restoration to normal values does not occur consistently after cART is implemented. Approximately 33% of patients who receive effective ART may experience persistently low levels of CD4 lymphocytes, despite the presence of virological suppression and long-term duration of therapy [19]. Incomplete CD4 recovery may be predicted by the presence of a profoundly low CD4 count (<200 cells/mm^3) at the time of initiating ART [20,21]. This is a manifestation of HIV-induced exhaustion or destruction of lymphoid tissue [22], loss of regenerative potential in the T-lymphocytic lineage and thymic dysfunction [23]. The cumulative viraemia that occurs during antiretroviral therapy, is proven to be a strong independent risk factor for developing HIV-associated lymphoma [24]. Survival outcome comparison with the HIV negative group demonstrated that HIV+ patients with CD4 counts <100 cells/mm^3 experienced a significantly inferior median OS outcome of 149 days (95% CI 44–254) versus 825 days (95% CI 309–1341) [$p = 0.04$] and a significantly higher motality hazard by univariate regression analysis [HR 3.08, 95% CI 1.16–8.17, $p = 0.02$]. The positive influence of c-ART is depicted in this study by the viral load being LDL for 30% (23/78) of the HIV+ DLBCL patients. SA has the largest ARV program worldwide and this country is currently striving to roll-out ARV therapy effectively.

Concomitant conditions [infections] (CCIs) included sepsis, tuberculosis, hepatitis B infection and herpes viral infections. CCIs were documented in 24 HIV seropositive patients and these were not significantly associated with CD4 counts <100 or ≥100 cells/mm^3 ($p = 0.61$ Chi-square test). When CCIs were present, an inferior median OS occurred in the HIV+ patients (154 days 95% CI 85–223, $p = 0.01$) and CCIs predicted a significantly inferior overall survial by multivariate regression analysis (HR 4.01, 95% CI 1.86–12.20, p = 0.02).

The treatment of HIV-associated NHL is steadily evolving [25-27]. Thirty-four patients, of which 22 were HIV+ and 5 had CD4 counts <100 cells/mm^3, received immunotherapy due to cost-limiting factors and unclarity regarding the role of rituximab in HIV+ DLBCL patients, given the increased risk of infection in patients with low CD4 counts. Rituximab treatment demonstrated proclivity towards a reduced mortality hazard by multivariate analysis (HR 0.04, 95% CI 0.00–1.66, $p = 0.09$).

Our study substantiates that HIV-associated non-Hodgkin lymphomas have a strong tendency towards an advanced stage at presentation [5,28-30]. A significantly advanced stage of lymphoma (i.e., Ann Abor III-IV, 87%, $p = 0.04$) and a predominance of IPI scores of 3-5 (75%) were evident. The latter was associated with an inferior median OS ($p = 0.01$) and hereby aligns with several other studies [31-33].

There was diffuse expression of CD20 in 99% (92/93) of HIV+ DLBCLs. One HIV+ DLBCL displayed limited expression of CD20, i.e., 20%, with diffuse expression of CD79a and PAX5. When CD20 immunoexpression is not present in a DLBCL which displays immunoblastic or anaplastic features, other pan-B-cell markers such as CD79a or PAX5, markers of terminal B-cell differentiation such as CD138 or Blimp1, LCA and in situ hybridisation investigations, such as kappa and lambda, are usually required to reliably distinguish DLBCL from plasmablastic lymphoma (PBL) or myeloma with anaplastic features. Herein, HIV+ DLBCL demonstrated CD10, bcl-6, MUM1 and BCL2 immunoreactivity in 44%, 85%, 66% and 41%, respectively. Co-expression of MUM1 and bcl-6 occurred in 53% of these tumours. By utilising the Hans et al [14] defined IHC COO, GC and NGC subtypes were present in 53% and 47%, respectively of the HIV+ DLBCLs. The median OS of the GC subtype was 398 days (95% CI 0–1083) and the NGC subtype was 105 days (95% CI 44–166), [$p = 0.10$]. This COO distribution is similar to the findings of several other HIV+ DLBCL studies in which significant differences in the overall survival outcome were also not demonstrated [16,34,35]. HIV+ DLBCL demonstrated significantly higher tumour proliferation indices (i.e., Ki-67 ≥90% in 85% of the cases [$p = 0.01$]) and there was a significant association of the double expression of BCL2 and c-MYC proteins with the NGC immunophenotype, [$p = 0.002$]. However, DE did not significantly impact the survival outcome in our study (HR 9.48, 95% CI 0.23–10.77, $p = 0.24$).

c-MYC protein expression occurs in 13-64% of DLBCL. In HIV+ DLBCLs c-MYC expression occurs more frequently, than in HIV- DLBCL, and is associated with increased mortality [46]. Expression of c-MYC protein tends to correlate with cellular proliferation, as an immediate early response to mitogenic influences. A gain of c-MYC function is associated with enhanced cell growth and proliferation [36-41]. c-MYC expression (≥40%) occurred in 58% of HIV+ DLBCLs and this was significantly associated with a SS appearance ($p = 0.03$) and a Ki67 proliferation index ≥90% ($p = 0.01$). However, c-MYC expression did not influence the median OS (c-MYC+ 325 days 95% CI 45–605 and c-MYC- 228 days 95% CI 143–313 [$p = 0.86$]).

Increased *MYC* gene copy numbers in DLBCL may adversely impact the survival outcome [8]. *MYC* amplification (i.e., ≥5 copies), which is infrequently documented in DLBCL, and c-MYC protein expression are associated with unfavourable overall survival and progression free survival [8,42]. Low-level gains of *MYC* are identified more frequently in DLBCL, i.e. 3-83% [8–13,43], and may result in higher levels of messenger ribonucleic acid expression [9]. However, it is questionable whether *MYC* gains in DLBCL exert a negative impact on the overall survival [11,44]. We identified a low-level increase in *MYC* gene copy numbers (i.e., 2.22–3.6 per tumour nucleus) in 57% of HIV+ DLBCLs and c-MYC protein expression varied from 40 to 80% within 83% of these tumours (Table 3). Although the median OS was shorter when increased *MYC* copy numbers were present (105 days, 95% CI 0-391) in the HIV+ group, a statistically significant difference was not evident when compared with the median OS when *MYC* copy numbers were not increased (752 days, 95% CI 0-1635, p = 0.52). Notably, our study contributes the presence of C8 polysomy (Fig. 2), *MYC* gene clusters (Fig. 3) and concurrent *MYC* rearrangement with increased *MYC* gene copies in HIV+ DLBCL. Unsuccessful dual-colour CISH was mainly due to technical challenges encountered with the autostainer instrument. Several months after the CISH staining occurred, signals had not faded within the tissue sections, as storage of the CISH-stained slides occurred away from direct sunlight.

MYC aberrations in HIV+ DLBCL are associated with less favourable survival outcomes [45]. *MYC* rearrangement was confirmed in 12% of HIV-associated DLBCLs by FISH, performed at the time of the initial diagnosis. *MYC* aberrations (i.e., increased *MYC* gene copy numbers and/or *MYC* rearrangement) were evident in 47% of the HIV+ DLBCLs and the presence thereof did not significantly influence the median OS (*MYC*+ 155 days 95% CI 0–356 and *MYC*- 154 days 95% CI 53–256, $p = 0.67$). The hazard ratio of 1.23 (95% CI 0.05–5.67), for *MYC* aberrations by multivariate analysis, was also not significant ($p = 0.89$).

5. Conclusion

Herein we demonstrate that HIV+ DLBCL is typified by a younger age at presentation, advanced stage of disease, a predominance of IPI scores 3-5, high tumour proliferation indices and double expression of c-MYC and BCL2 in the context of a NGC COO immunophenotype. Low-level increase of *MYC* gene copy numbers occurs more frequently than *MYC* rearrangements. Rarely, *MYC* gene clusters and concurrent *MYC* rearrangement with increased *MYC* gene copies are detected. Inferior median OS occurs when CD4 counts are <100 cells/mm^3 and when IPI scores are 3–5. Concomitant infections negatively impact the overall survival. The influence of rituximab immunotherapy on the survival outcome of HIV+ DLBCL patients is currently being investigated within prospective clinical trials at our institute.

5.1. Study limitations

The delayed presentation of patients to health care facilities or the lack of timeous referral of the patients to the Clinical Haematology Unit may have contributed to the advanced stage of lymphoma at presentation. Due to the retrospective nature of this study, clinical data of all the

patients were not found at the Clinical Haematology Unit archives. Some of the patients might have demised prior to their referral to the unit and others might have sought lymphoma treatment at other medical institutions. During the study timeframe, there was variation in the treatment regimens for DLBCL and a limited number of patients received rituximab immunotherapy. Non-compliance on cART and other therapeutic agents are also potential limiting factors. There were limited numbers of HIV- DLBCL patients in this study. Some patients were inadvertently excluded from this study due to a paucity of diagnostic tissue. Due to technical challenges with the autostainer instrument and a paucity of diagnostic tissue within several tissue blocks, CISH was not performed on all the tumours. CISH was deemed unsuccessful on 5 tumours due to absent or insufficient *MYC* signals.

Ethics

Institutional ethics approval of this study was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (certificate numbers: M151017 and M2010122).

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