





RESEARCH PAPER

Checkpoint CD47 expression in classical Hodgkin lymphoma

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Summary

The glycoprotein CD47 regulates antiphagocytic activity via signal regulatory protein alpha (SIRPa). This study investigated CD47 expression on Hodgkin and Reed–Sternberg (HRS) cells in the classical Hodgkin lymphoma (cHL) tumour microenvironment and its correlation with prognosis, programmed-death (PD) immune markers, and SIRPa⁺ leukocytes. We conducted immunohistochemistry with CD47 and SIRPa antibodies on diagnostic biopsies (tissue microarrays) from cHL patients from two cohorts ($n = 178$). In cohort I ($n = 136$) patients with high expression of CD47 on HRS cells ($n = 48$) had a significantly inferior event-free survival [hazard ratio (HR) = 5.57; 95% confidence interval (CI), 2.78–11.20; $p < 0.001$] and overall survival (OS) (HR = 8.54; 95% CI, 3.19–22.90; $p < 0.001$) compared with patients with low expression ($n = 88$). The survival results remained statistically significant in multivariable Cox regression adjusted for known prognostic factors. In cohort II ($n = 42$) high HRS cell CD47 expression also carried shorter event-free survival (EFS) (HR = 5.96; 95% CI, 1.20–29.59; $p = 0.029$) and OS (HR = 5.61; 95% CI, 0.58–54.15; $p = 0.136$), although it did not retain statistical significance in the multivariable analysis. Further, high CD47 expression did not correlate with SIRPa⁺ leukocytes or PD-1, PD-L1 and PD-L2 expression. This study provides a deeper understanding of the role of CD47 in cHL during an era of emerging CD47 therapies.

KEYWORDS

Hodgkin lymphoma, CD47, lymphoma, SIRPa, tumour markers

INTRODUCTION

Classical Hodgkin lymphoma (cHL) is a B-cell-derived malignancy characterised by a tumour microenvironment (TME) composed of a few malignant Hodgkin and Reed–Sternberg (HRS) cells, surrounded by leukocytes consisting of T cells, B cells, mast cells, macrophages, plasma cells, eosinophils and mesenchymal stromal cells.^{1–3} Although progress has been made in the treatment of cHL, the prognosis of patients with disease relapse is still poor.⁴

Programmed-death (PD)-1 inhibitors are approved to treat progressive and relapsed cHL,^{6,7} and higher expression of programmed-death-ligand (PD-L)-1 in HRS cells predicts superior response rates and survival outcomes.^{7,8} However, PD-1 inhibitors have uncertain response durability, and 65%–87% of patients are objective responders.^{7,9–11} Hence, there is an unmet need to explore different tumour immune escape mechanisms in the TME of cHL and identify new checkpoint targets.⁶

The cluster of differentiation 47 (CD47) is a transmembrane glycoprotein that transduces a checkpoint signal to

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phagocytic cells, delivering a 'do not eat' signal via signal regulatory protein alpha (SIRPa).¹² CD47 is presumably expressed by most cells in the body that need protection from phagocytosis. In haematological and solid malignancies, overexpression of CD47 on tumour cells is associated with poor survival.^{12–14} Targeting the CD47–SIRPa axis checkpoint is currently investigated in clinical trials across several haematological malignancies, including cHL,^{15,16} with encouraging results in non-Hodgkin lymphoma.¹⁷

While one report recently suggested that CD47 overexpression in HRS cells is common among cHL cases,¹⁸ no studies have explored the long-term prognostic and clinical implications of high CD47 tumour expression or its correlation with different immune-cell markers, including PD-1 and PD-L1 in cHL. This study aimed to investigate the prognostic value of CD47 expression in the TME in cHL in two independent cohorts. In addition, we investigated the association of CD47 expression with SIRPa-positive leukocytes, PD-1, PD-L1, PD-L2 and several other immune cells.

MATERIALS AND METHODS

Ethical considerations

The study was granted ethical approval by the national and regional Ethical Review Boards: Dnr 99–154 for, Dnr 2014/233, Dnr 01–367 and Dnr 2014/020.

Study cohort

The study subjects of the first cohort were part of the Scandinavian Lymphoma Aetiology (SCALE) study, a large case-control study conducted in Denmark and Sweden between 1999 and 2002, described in detail elsewhere.¹⁹ Expert haematopathologist revisited all cases and classified histological subtypes in agreement with the WHO classification.²⁰ The patient flow chart is provided in Figure 1. Patients' medical records were reviewed for clinical variables, administered treatment and pre-treatment blood test

results, that is, erythrocyte sedimentation rate (ESR), haemoglobin and albumin.

Clinical stage was defined according to the Ann Arbor classification.^{21,22} Epstein–Barr virus (EBV) status was previously determined for this cohort by using EBV latent membrane protein (LMP)-1 (Ventana Benchmark) and *in situ* hybridisations for EBV-encoded small RNAs (EBER).²³ The proportion or count per high-power field for the following immune cells and checkpoint markers was analysed with immunohistochemistry and described in detail elsewhere: PD-1, PD-L1 and PD-L2,²⁴ CD138⁺ plasma cells,²⁵ tryptase⁺ mast cells,²⁶ granzyme B⁺-activated lymphocytes,³ forkhead box P3⁺ regulatory T cells (FOXP3⁺ Tregs)³ and CD68⁺ macrophages.³

The second cohort comprised patients from a biobank programme, UCAN (Uppsala Umeå Comprehensive Cancer Consortium), which has collected data and created a biobank with blood and tissue samples that include samples from lymphoma patients since 2010. All patients were diagnosed with cHL; one patient was diagnosed in 1986 and was recruited retrospectively in 2011 after first relapse. Included cases in current study were confined to patients ($n = 42$) with available diagnostic biopsies for tissue microarray (TMA) construction. Data variables for cohort two as well as EBV analysis were retrieved similarly for the SCALE cohort.

Western blot material and preparation of tissue lysates

Fresh frozen tumour tissues from five patients (UCAN cohort) and lymph node tissues (designated normal tissues) from two healthy individuals diagnosed with benign reactive lymphadenopathy were analysed by western blot. In addition, further clustered regularly interspaced short palindromic repeats (CRISPR) gene-edited cell line lysates HEK 293 CD47T KO and HEK 293 T wt cell (both from Abcam, Cambridge, United Kingdom) were used as negative and positive controls respectively.

The tumour tissues and normal tissues were mixed with lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8, 1% Triton X-100, 0.1% sodium deoxycholate,

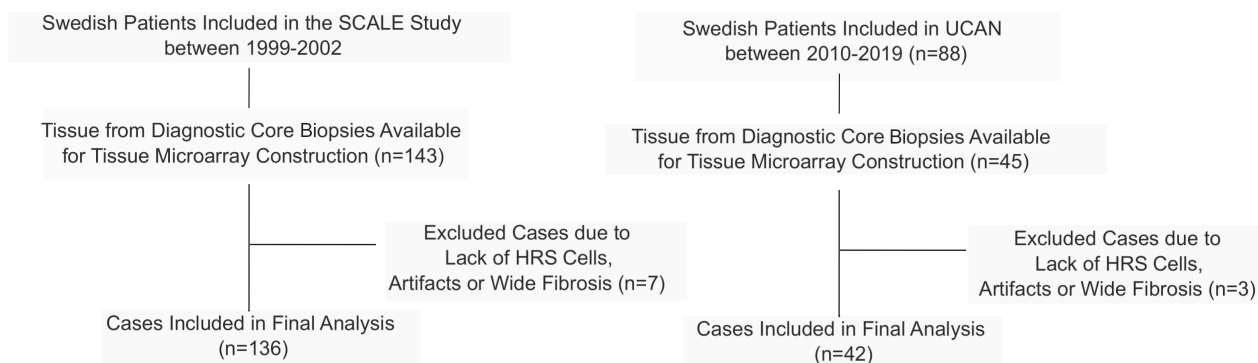


FIGURE 1 Flowchart of study participants cohort one (left) and cohort two (right). SCALE = SCAndiavian lymphoma Aetiology study, HRS, Hodgkin and Reed–Sternberg cells; UCAN, Uppsala Umeå comprehensive cancer consortium biobank programme.

a protease inhibitor (Roche Complete Mini, Merck KGaA, Darmstadt, Germany)], and zirconium beads (ZrOB20-RNA, 2 mm in diameter, Next Advance Inc.). The ratio of sliced tissue mass:volume of lysis buffer:zirconium beads weight was 1:4:2. A Bullet Blender device (BBX24B-CE, Next Advance Inc.) was used for homogenisation according to the manufacturer's recommendation for lymphatic tissue. Centrifugation at 13 000 rpm for 10 min at 4°C was performed on the homogenised tissues, and the supernatant was transferred to new tubes. The total protein concentration of tissue lysate was measured by Pierce™ BCA Protein Assay kit (ThermoFisher).

Western blot and intensity values

A total of 40 µg from each sample was mixed with Tris–Glycine SDS sample buffer (Invitrogen, Fisher Scientific, Sweden) and separated by electrophoresis on a NuPAGE 10% Bis–Tris Gel (Invitrogen, Fisher Scientific, Sweden). Proteins were transferred to a nitrocellulose membrane using iBlot Transfer Stack mini according to the manufacturer's instructions (Invitrogen). Membranes were blocked for 1 h in Intercept [Tris-buffered saline (TBS)] blocking buffer (Li-Cor Biosciences GmbH, Germany) and then incubated with CD47 (LS-C658462), a polyclonal antibody (LifeSpan Biosciences, Seattle, WA, USA), diluted 1:100 in blocking buffer and incubated at 4°C overnight. Membranes were washed three times for five minutes washing in 1× TBS with 0.05% Tween 20 (Sigma Aldrich, Merck KGaA) and incubated with IRdye 800CW donkey anti-Rabbit IgG secondary antibody (Li-Cor Biosciences) diluted 1:15 000 in blocking buffer for 1 h at room temperature. After a final wash in TBS, the protein bands were visualised using a Li-Cor Odyssey scanner (Li-Cor Biosciences).

The intensity values of protein bands were calculated using ImageJ (Java-based image-processing and analysis software; <http://rsb.info.nih.gov/ezproxy.its.uu.se/nih-image/>). An area of the same size, in each western blot band and the lightest area of the image, was used for analysis. Then, the mean value of reflected light per pixel was subtracted from the lightest area of the image, giving a mean blackness per pixel (bpp) value (i.e., intensity value of the band).

Tumour samples and immunohistochemistry

Diagnostic tumour tissue (formalin-fixed paraffin-embedded) from patients with cHL was subject to TMA construction according to standard technique (4 µm thin sections).²⁷ Immunohistochemistry was performed using the Dako automated staining system (Dako, Santa Clara, CA, USA) at the Department of Clinical Pathology at Uppsala University Hospital, Sweden. Normal tonsils, lymph nodes and liver tissue were used as controls.

Immunohistochemical double stains for CD47/paired box protein 5 (PAX-5) were performed; CD47 was recognised using a polyclonal antibody, LS-C658462 (LifeSpan Biosciences) diluted 1:1000. PAX-5 was identified with a

monoclonal antibody, M7307/DAK-Pax5 (Dako), diluted 1:100. A MACH2 DS1 detection kit (Biocare Medical) was used for detection, diaminobenzidine (DAB) chromogen detecting CD47 and Warp Red chromogen detecting PAX5. SIRPa was identified using a monoclonal antibody, sc-17 803/A-1 (Santacruz Biotech) and EnVision FLEX+ High pH Kit (Agilent). The sections were counterstained with Mayers haematoxylin (Histolab Products AB).

Scoring and assessments

The biopsy cores were previously compared with whole tissue sections regarding the distribution of tumour-infiltrating leukocytes with good agreement.²⁸ Between two and four core biopsies with an area of 1 mm² were analysed for each case by two separate researchers, PH (senior haematopathologist) and AG. The visualisation functions of an image analysis software (Visiomorph, Visiopharm, Hørsholm, Denmark) were used, and HRS cells were identified manually. Cases that lacked HRS cells or had major fibrosis, discolouration or artefacts were omitted (Figure 1). Since all HRS cells expressed CD47 with at least weak membranous and cytoplasmic intensity, a simplified variant of the Allred method was used for the scoring.²⁹ Cases with HRS cells expressing CD47 with weak or intermediate intensity (corresponding to an Allred score of 6–7) were classified as cases with low expression of CD47 on HRS cells. Cases with HRS cells expressing CD47 with strong intensity compared to adjacent cells and a homogeneous staining pattern (corresponding to an Allred score of 8) were designated as cases with a high expression of CD47 on HRS cells (Figure 2). Cases containing both HRS cells with low and with high CD47 expression were classified according to the pattern expressed by most of the HRS cells. A few ambiguous cases were discussed between the two assessors to reach a consensus, with a high overall interobserver agreement. The SIRPa scoring assessment was only assessed in leukocytes due to negativity in all HRS cells. A SIRPa⁺ leukocyte proportions score was calculated by the image software analysis fitted to divide the number of positive leukocytes with the total number of positive and negative leukocytes in 4–6 high-power fields (HPF), at 400×, 0.0625 mm².

STATISTICAL ANALYSES

Survival functions were analysed with the Kaplan–Meier method and associated log-rank tests. Cox regression was used for analysing hazard ratios (HRs) with a 95% confidence interval (95% CI) and Wald tests. Time of event-free survival (EFS) was calculated from diagnosis until disease progression, tumour relapse, first-line treatment failure due to any cause, or death from any cause. Time of overall survival (OS) was calculated from diagnosis until death from any cause. Considering the number of patients and events in the multivariable Cox regression models, five covariates were

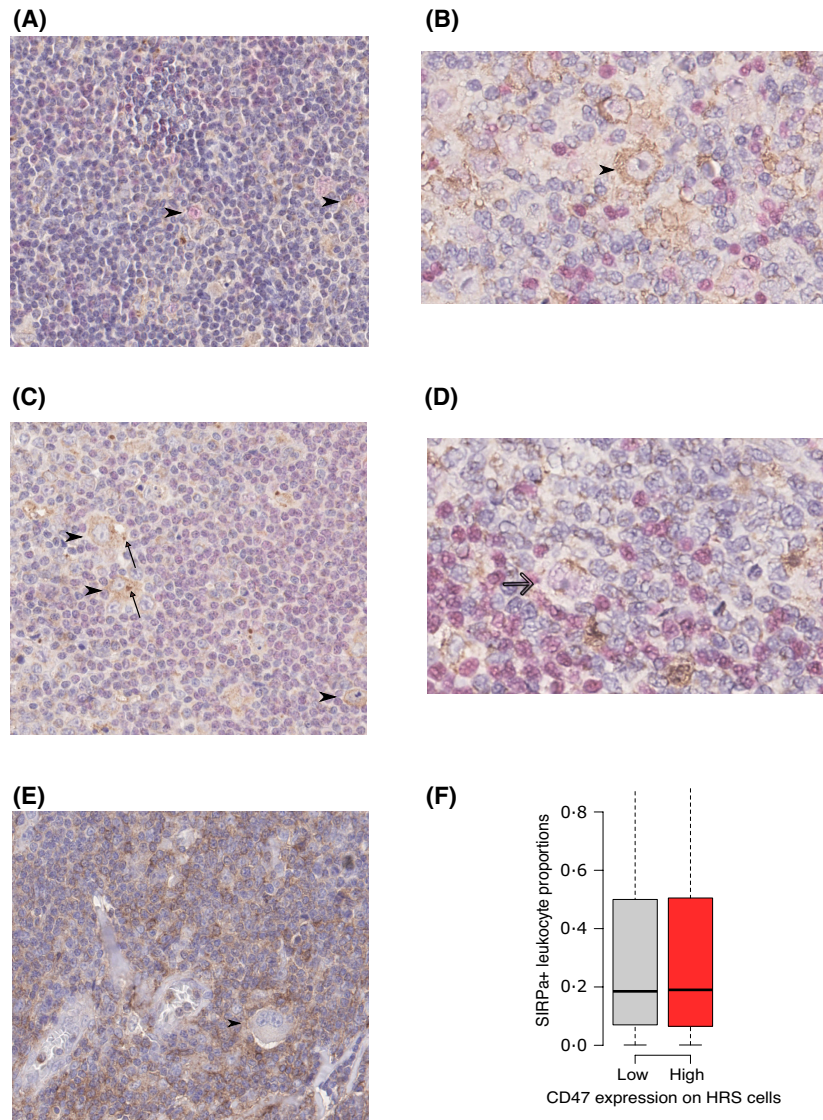


FIGURE 2 Immunohistochemical staining and correlations. (A) Immunohistochemical stains captured with light microscopy with original magnification 400 \times . The arrowheads indicate Hodgkin and Reed–Sternberg cells (HRS) with low CD47 expression and weak PAX5 staining. (B) Picture captured digitally from 400 \times magnification and further enlarged to 250%. Arrowhead indicates a case scored as high expression of CD47 on HRS cells, lacking the dot-like pattern in the Golgi area. (C) Arrowheads indicate three of several HRS cells with high CD47 expression. The arrows mark dot-like staining of the Golgi area. (D) Arrow indicates an HRS cell expressing partial CD47, categorised as low expression. (E) Immunohistochemical stains, original magnification 400 \times , with light microscopy for SIRPa⁺ leukocytes in the tumour microenvironment of classical Hodgkin lymphoma; a negative HRS cell is indicated by the arrowhead. (F) Boxplot for SIRPa⁺ leukocyte proportions in patients with high expression of CD47 on HRS cells (median = 0.19) compared to patients with low expression of CD47 on HRS cells (median = 0.185) (two-sample Wilcoxon test, $p = 0.83$) [Colour figure can be viewed at wileyonlinelibrary.com]

selected to avoid model overfitting. Included covariates had the strongest prognostic impact on EFS and OS (Table S1) or the most skewed distribution between groups compared. Assumptions of the proportional hazards were validated with Schoenfeld residuals. In addition, the nearest-neighbour (NN) method in the R-package *MatchIt* version 4.3.3 with calliper 0.2 was used for propensity score (PS) matching³⁰ to generate balanced and comparable groups. In PS matching, each observation is paired with a control unit with the closest propensity score. The calliper of 0.2 sets the acceptable distance for any match. Any observations outside the distance of the calliper are excluded.³⁰ The covariate selection in the PS matching model was the same as with the Cox regression.

Tests for independence of categorical variables were calculated with the chi-squared test or Fischer's exact test. Normality was analysed with the Shapiro–Wilk test. The two-sample Wilcoxon test (Wilcoxon–Mann–Whitney test) was used to evaluate the association between a categorised variable and a continuous variable with a non-normal distribution. Clinicopathological correlations, including associations with SIRPa, were investigated on the SCALE cohort. A Spearman test (rho coefficient) was used for determining correlations between two non-normal continuous variables. R version 3.2.2 (<https://cran.r-project.org/>) was used for all statistical calculations, and two-sided p -values less than 0.05 were defined as statistically significant.

Cut-offs

The CD47 scoring method yielded a predefined two-level categorised (high/low) variable during scoring and was treated as such (dichotomous) in all statistical analyses. Hence no further statistical cut-offs were utilised. SIRPa⁺ leukocyte scoring outcome was treated as a continuous variable in univariate Cox regression and receiver operating characteristic (ROC) curves. The area under the curve (AUC) was used to determine its accuracy to predict survival outcomes. Different cut-offs were also examined with the Kaplan–Meier method for SIRPa⁺ leukocytes to identify any significant prognostic influence.

RESULTS

Patient characteristics and treatment

Complete baseline characteristics are available in Table 1. The baseline characteristics of the patients included in the current study were comparable to the characteristics of their original cohorts (Table 1), as were the survival outcomes between the cohorts examined (Figure S1). First-line chemotherapy protocols were mainly ABVD (doxorubicin, bleomycin, vinblastine

and dacarbazine) or BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone) as recommended by the Swedish national guidelines.^{5,31} Radiotherapy was given after chemotherapy to patients with clinical stage I–IIA and individual patients with advanced disease (\geq IIB). Some patients (17% SCALE and 13% UCAN) were treated with outdated first-line regimes (Table S2). Relapsed patients who reached complete remission after salvage therapy were treated with autologous stem cell transplantation when eligible.

Western blot

We observed the 50 kDa band of the CD47 protein on the western blot, which confirmed the specificity of the CD47 antibody binding (see Figure S2). In addition, we found no staining from the loaded HEK293T knock-out (CD47-negative) cell lysate sample, and we could confirm a band for CD47 in the HEK293T wild-type cell lysate (see Figure S2). Further, the two-control tissue from reactive lymph nodes showed lower CD47 intensity bands than the five patient tissue samples. However, there was no correlation between band intensity readings and CD47 scoring on HRS cells, as expected. The bands represented

TABLE 1 Base-line demographics

	Full UCAN cohort (n = 88)	Current UCAN cohort (n = 42)	Full SCALE cohort (n = 571)	Current SCALE cohort (n = 136)
Age (years): median (range)	41 (12–85)	39.50 (12–85)	35 (17–74)	38 (18–74)
Age \geq 60 (n)	23 (26%)	8 (19%)	97 (26%)	21 (15%)
Male sex (n)	58 (66%)	29 (69%)	302 (81%)	81 (60%)
Follow-up time (y); median (range)	4.50 (0.36–26)	5.2 (0.66–26)	13.9 (0.37–15.9) Missing = 242	13.8 (0.59–15.9)
5-year OS probability	85%	90%	91% Missing = 242	90%
2-year EFS probability	84%	86%	88% Missing = 266	90%
Advanced stage (n) (IIB–IVA)	56 (64%)	21 (50%)	180 (55%) Missing = 243	77 (57%)
IPS \geq 2 (n)	64 (83%) Missing = 11	26 (72%) Missing = 6	108 (54%) Missing = 371	59 (57%) Missing = 33
BEACOPP at first-line (n)	14 (16%) Missing = 2	4 (10%) Missing = 1	64 (19%) Missing = 237	31 (23%) Missing = 1
ABVD at first-line (n)	55 (63%) Missing = 1	31 (76%) Missing = 1	206 (62%) Missing = 237	81 (60%) Missing = 1
EBV ⁺ cases	16 (25%) Missing = 23	10 (24%)	130 (29%) Missing = 75	32 (24%) Missing = 3
Nodular Sclerosis	53 (63%) Missing = 4	31 (74%)	407 (77%) Missing = 47	97 (71%)

For assigned treatment regimens, patients were categorised according to the regimens that constituted the majority of all cycles; for example, patients receiving two ABVD followed by six BEACOPP were classified as BEACOPP patients. All patients received six cycles of treatment as standard. Shorter regimens were always associated with EFS events, such as toxicity, progression or death. Advanced stage, according to Ann Arbor; age, age at diagnosis.

Abbreviations: ABVD, doxorubicin, bleomycin, vinblastine and dacarbazine); BEACOPP, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone; EBV, Epstein–Barr virus; EFS, event-free survival; IPS, international prognostic index; OS, overall survival; SCALE, SCAndiavian Lymphoma Etiology study; UCAN, Uppsala Umeå Comprehensive Cancer Consortium biobank programme; WHO, performance status according to the World Health Organisation's Eastern Cooperative Oncology Group (ECOG).

whole tissues, with most cells being non-malignant in patients' tissues, while the scoring of CD47 was confined to HRS cells.

Immunohistochemistry

All HRS cells expressed CD47, observable mainly with membranous and cytoplasmic staining (Figure 2). In the SCALE cohort, 48 patients (35%) were categorised as having a high CD47 expression, and 88 patients (65%) had a low expression. In the UCAN cohort, 16 patients (38%) were classified as having HRS cells with a high CD47 expression and 26 patients (62%) as having low CD47 expression on HRS cells. Cases with a high CD47 expression had intense, homogeneous staining, often with dot-like staining of the Golgi area. Cases with low expression had a weak, often heterogeneous cytoplasmic staining and no staining of the Golgi (Figure 2). The staining for SIRPa⁺ leukocytes was both membranous and cytoplasmic (Figure 2E). HRS cells lacked antibody staining for SIRPa, and the morphologies of positive leukocytes varied. We did not observe any association between high CD47 expression on HRS cells and proportions of SIRPa⁺ leukocytes (Figure 2F: $p = 0.83$). There was no apparent correlation of

co-expression between PAX-5-positivity and CD47 expression on HRS cells.

Correlation of study markers with clinicopathological features

Patients with a high expression of CD47 on HRS cells had significantly lower haemoglobin ($p < 0.01$), lower albumin ($p = 0.01$), higher age ($p = 0.01$) and higher ESR ($p < 0.01$) than patients whose HRS cells had a low expression of CD47 (Table 2). We did not find any statistically significant association between CD47 expression and PD-1, PD-L2 or PD-L1 expression (Table 2). Furthermore, there was no statistically significant difference between patients with high and low expression of CD47 on HRS cells regarding clinical stage, histological subtype of cHL, sex, tumour EBV status or presence of B symptoms (Table S3).

Increasing SIRPa⁺ leukocyte proportions correlated with increasing PD-L1⁺ leukocytes ($\rho = 0.40$; $p < 0.001$), PD-L1⁺ HRS cells ($\rho = 0.22$; $p = 0.02$) and decreasing blood lymphocyte count ($\rho = -0.20$; $p = 0.03$), Table 2. EBV-positive cases ($n = 31$) had higher SIRPa⁺ leukocyte proportions (median = 0.32, $p = 0.001$) compared with EBV-negative cases

TABLE 2 Full correlation analysis in the SCALE cohort

Predictor variables	CD47 expression on HRS cells categorical			SIRPa ⁺ leukocytes continuous		
	Low median of predictor variable	High median of predictor variable	<i>p</i> -Value	Rho	<i>p</i> -Value	<i>n</i>
Clinical features						
ESR (mm/h)	27.50	55.50	<0.01	0.02	0.90	124
Age (years)	30.50	37.00	0.02	-0.06	0.52	136
White blood cell count (count × 10 ⁹)	8.50	9.20	0.14	-0.02	0.81	131
S-Albumin (g/dl)	40.00	37.50	0.01	-0.08	0.37	122
Haemoglobin (g/l)	113	117	<0.01	0.06	0.53	121
Lymphocyte count (count × 10 ⁹)	1.50	1.70	0.76	-0.20	0.03	120
Immune cells in the tumour microenvironment						
Plasma cells (proportion)	0.01	0.01	0.57	-0.12	0.21	118
CD68 ⁺ macrophages (proportion)	0.07	0.07	0.91	0.12	0.18	123
Tryptase ⁺ mast cells (count)	23.00	31.50	0.28	0.14	0.10	136
FOXP3 ⁺ Tregs (proportion)	0.06	0.05	0.27	-0.11	0.22	121
Granzyme B ⁺ lymphocytes (proportion)	0.03	0.03	0.62	0.04	0.67	121
Programmed death ligands and receptor						
PD-L1 ⁺ leukocytes (proportion)	0.15	0.20	0.15	0.40	<0.01	122
PD-L1 ⁺ HRS cells (proportion)	0.45	0.55	0.71	0.22	0.02	122
PD-L2 ⁺ leukocytes (proportion)	0.01	<0.00	0.29	-0.02	0.87	120
PD-L2 ⁺ HRS cells (proportion)	<0.00	<0.00	0.36	-0.13	0.20	120
PD-1 ⁺ leukocytes (proportion)	0.03	0.02	0.35	-0.01	0.92	123

Column one shows the predictors. Columns two and three show the median of the predictors in the corresponding CD47 scoring group. The third column shows *p*-values retrieved with a two-sample Wilcoxon test. Column four shows the rho correlation coefficient with corresponding *p*-values in column 5, retrieved with the Spearman test. *n*, number of patients included in the analysis, missing values due to lack of data for predictors or dependent values; SIRPa⁺ leukocytes, proportions scored in the tumour microenvironment. Abbreviations: ESR, erythrocyte sedimentation rates; FOXP3, Forkhead box P3; HRS, Hodgkin and Reed–Sternberg Cell (HRS); PD, programmed death; PD-L programmed-death-ligand.

($n = 131$, median = 0.15). SIRPa⁺ leukocyte proportions lacked significant correlation with clinical stage ($p = 0.72$), B symptoms ($p = 0.98$), male sex ($p = 0.16$) and histological cHL subtype ($p = 0.91$).

Survival analysis

Baseline demographics for groups compared stratified by CD47 expression levels for the SCALE and the UCAN cohorts are available in [Table S3](#) and [Table S4](#). In the SCALE cohort ($n = 136$), patients with a high expression of CD47 on HRS cells ($n = 48$) had an inferior EFS [[Figure 3](#): hazard ratio (HR) = 5.57; 95% CI, 2.78–11.20; $p < 0.001$] and OS ([Figure 3](#): HR = 8.54; 95% CI, 3.19–22.90; $p < 0.001$) compared with patients with a low expression of CD47 on HRS cells ($n = 88$). Survival outcomes remained statistically significant in multivariable Cox regression adjusted for well-known prognostic covariates including age > 45 years, advanced stage, albumin < 4 g/dl, haemoglobin < 105 g/l, and white blood cell count (WBC) $\geq 15 \times 10^9$. The adjusted multivariable Cox regression analysis for EFS and OS remained significant with different cut-offs for age, >55 years and >65 years, and when age was treated as a continuous variable ([Table S5](#)). Further, the adverse prognostic impact of high expression of CD47 on HRS cells in the SCALE cohort was observed in subgroups of patients treated with modern first-line lymphoma therapy (ABVD/BEACOPP) and in analyses using PS matching, comparing groups balanced regarding the same covariates as in the Cox regression model [age > 45 years (and different cut-offs), advanced stage, albumin < 4 g/dl, haemoglobin < 105 g/l, and WBC $\geq 15 \times 10^9$; [Table S6](#)]. Analysed as a continuous variable, SIRPa⁺ leukocytes had a low AUC for OS outcome (0.53) and did not have any significant prognostic value on EFS (HR = 0.91; 95% CI, 0.27–0.3.13; $p = 0.89$) and OS (HR = 1.58; 95% CI, 0.38–6.58; $p = 0.53$) in univariate Cox regression. Further different SIRPa leukocyte cut-offs proportions, examined with the Kaplan–Meier method, did not significantly correlate with survival outcomes.

For the UCAN cohort ($n = 42$), high CD47 expression on HRS was a predictor for inferior EFS (HR = 5.96; 95% CI, 1.20–29.59; $p = 0.029$) in univariate Cox regression but lost statistical significance for impact on OS (HR = 5.61; 95% CI, 0.58–54.15; $p = 0.136$). High CD47 expression on HRS cells was not statistically significantly associated with EFS ($p = 0.07$) and OS ($p = 0.99$) in multivariable analyses adjusting for advanced stage, albumin < 4 g/dl, haemoglobin < 105 g/l, white blood cell count (WBC) $\geq 15 \times 10^9$ and age. Further, in the subgroup of patients treated with ABVD or BEACOPP in the UCAN cohort ($n = 35$), the survival implications did not reach significance for EFS ($p = 0.99$) and OS ($p = 0.10$).

Finally, when we examined all patients from both cohorts ($n = 178$), we found that high CD47 expression on HRS cells correlated with inferior EFS (HR = 5.78; 95% CI, 3.05–10.91; $p < 0.001$) and OS (HR = 8.10; 95% CI, 3.28–20.00; $p < 0.001$), retaining significance in multivariable analysis and when patient subgroup was confined to ABVD/BEACOPP treatments, similar as for the SCALE cohort ([Table S7](#)).

DISCUSSION

The main finding of this study is the observed different expression of CD47 on HRS cells between patients and its adverse prognostic implications. Interestingly CD47 expression did not correlate with several investigated immune cells and programmed-death checkpoint markers. Moreover, SIRPa⁺ leukocytes lacked a significant correlation with CD47 expression levels or EFS and OS. However, the presence of SIRPa⁺ leukocytes correlated with increased proportions of PDL-L1⁺ leukocytes and PD-L1⁺ HRS cells. These findings add to current understanding of the role of CD47 in cHL during an era of increased need for personalised medicine and ongoing trials targeting CD47 in several malignancies.

A recent study by López-Pereira *et al.*¹⁸ found that the 'over-expression' of CD47 varies among cHL cases and that patients with overexpression of CD47 on HRS cells had a predominant diffuse granular cytoplasmic staining pattern similar to the dot-like Golgi pattern observed in the current study. Although only 16 cases were included in the study by López-Pereira *et al.*,¹⁸ they scored 27% of the cases as 3+ for CD47 expression on HRS cells and 63% as 1+ or 2+, which is highly comparable to results in the current study in which 35% were scored as cases with high expression of CD47 on HRS cells. These differences in proportions in low/high HRS cell CD47 expression most likely reflect random variation between study populations and different scoring methods. In the current study, high expression of CD47 on HRS cells was associated with inferior survival. It remained significant in relevant patient strata and adjusted multivariable analysis, suggesting that the CD47 checkpoint pathway is an essential mechanism for tumour surveillance escape in cHL even in the absence of CD47 inhibition. The findings of this study are consistent with the extensive evidence regarding the role of CD47 in cancer immune evasion and its association with inferior survival in several malignancies.^{12–14} Even though the primary mechanism for CD47 is to promote tumour survival through antiphagocytic regulation via SIRPa, several other mechanisms have been attributed to CD47, such as tumour migration via binding to integrins^{32,33} and increased proliferation via activation of intracellular signals like phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt).³⁴

In the current study, high expression of CD47 on HRS cells correlated with lower albumin, lower haemoglobin, higher ESR and higher age. A biological explanation for these associations is still uncertain. A link between associated inflammatory parameters and high expression of CD47 on HRS cells could be due to pluripotent cytokines that can both promote inflammation and upregulate CD47 in tumour cells.³⁵ The lack of correlation of CD47 protein expression and PD1, PD-L1 and PD-L2 was unexpected since a study has shown a correlation between PD-L1 mRNA and CD47 mRNA levels in leukaemic malignancies.³⁶ However, in the same study, no correlation between PD-L1 and CD47 protein expression was observed, consistent with current findings.³⁶

While CD47 is ubiquitously expressed, mainly myeloid cells are believed to express SIRPa.³⁷ We did not see any correlation between CD47 expression on HRS cells and SIRPa⁺

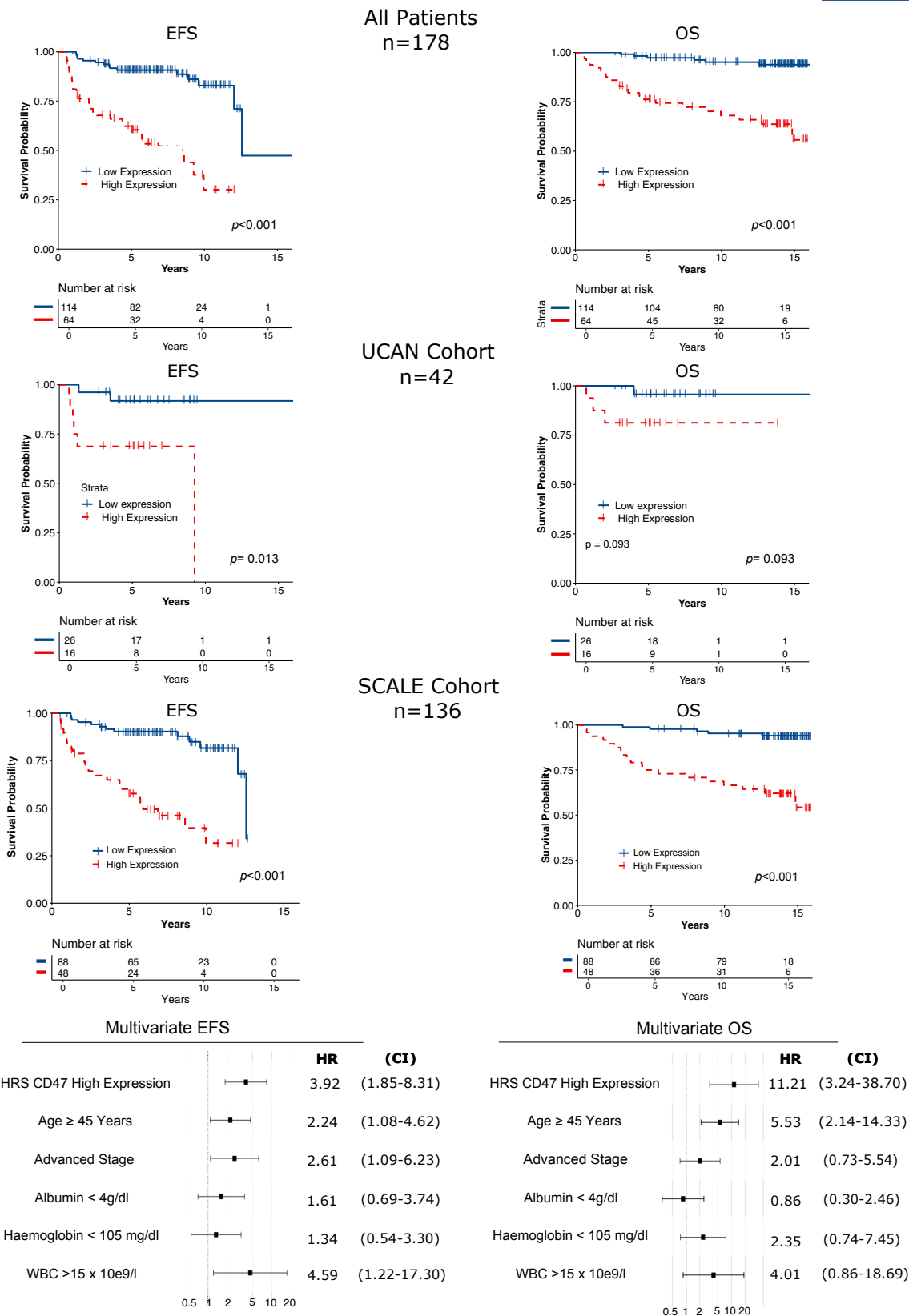


FIGURE 3 Survival analysis. Kaplan–Meier event-free survival (EFS) and overall survival (OS) plots with associated log-rank *p*-value comparing patients with high and low expression of CD47 on Hodgkin and Reed–Sternberg cells in all patients and the different cohorts. At bottom, multivariable Cox regression forest plot for EFS, for the SCALE cohort, 95% confidence interval (CI) for hazard ratios (HR) on the *x*-axis, for EFS, *n* = 112, 22 cases excluded due to missing data in covariates, number of events = 33. For overall survival (OS, *n* = 112), number of events = 22 [Colour figure can be viewed at wileyonlinelibrary.com]

leukocytes. However, we did not exclusively explore SIRP^a macrophages but rather the overall SIRP^a in all leukocytes. The association between SIRP^a and PD-L1 could be due to tumour necrosis factor (TNF) alpha, which can upregulate SIRP^a expression³⁸ and PD-L1.³⁹ Further, studies show that an increased EBV viral load can upregulate PD-L1,^{40–42} and a similar mechanism could be behind the correlation between EBV⁺ cases and increased proportion of SIRP^a leukocytes observed in the current study.

LIMITATIONS AND STRENGTHS

The retrospective nature of this observational study makes the results vulnerable to the effects of unbalanced and incomparable groups. However, we utilised PS matching⁴³ and traditional methods like Cox regression to assess the independent prognostic significance of CD47 expression. The results of the survival analyses in this study should be cautiously interpreted since the overall sample size ($n = 178$) is moderate, and due to a low number of patients, most likely did not show the same prognostic implications in the more modern cohort UCAN ($n = 42$). Moreover, there are general limitations in defining CD47 expression levels with immunohistochemistry, and next steps should be to utilise single-cell RNA sequencing as well as proteomics on isolated HRS cells via DEPArray™ technology and FACS^{44,45} to deepen our understanding of CD47 regulation in HRS cells. However, the sparse number of HRS cells in the TME of cHL makes an immunohistochemical methodology beneficial since it enables assessing the spatial and subcellular localisation of investigated protein. Thus, we could distinguish CD47 expression on HRS cells and non-malignant cells, which revealed that cases with high expression of CD47 on HRS cells showed, in most cases, a dot-like staining of the Golgi area (Figure 2B).

Moreover, the used CD47 antibody in this study has been validated in a previous study by López-Pereira *et al.*¹⁸ by double immunofluorescence co-expression of CD47 together with CD30 on HRS cells as with western blot in the current study. However, the western blot was performed on tissue lysate and could not work as a surrogate for CD47 expression scoring on HRS cells. Furthermore, two independent assessors conducted the immunohistochemical assessment, including a senior haematopathologist, with a high interobserver consistency regarding the assessment. A further strength of the study is the long-term follow-up of the cohort and that most of the patients had been treated with first-line lymphoma treatments still considered as contemporary approaches.^{5,31,46}

CONCLUSION

Introducing new checkpoint markers with prognostic impact is of high interest in cHL. This study showed that expression of CD47 on HRS cells varied between cases and had adverse

prognostic implications. The findings provide a broader insight into potential different tumour escape mechanisms in cHL during an era of personalised medicine and emerging CD47 therapies.

ACKNOWLEDGEMENT

We wish to give special thanks to Anders Berglund, PhD in medical epidemiology and biostatistics, and senior medical statistic consultant at Statistikakademien AB, Uppsala, for discussions and statistical calculations.

CONFLICT OF INTEREST

All authors declare no competing financial interests.

AUTHOR CONTRIBUTION

A.R. Gholiha planned the study, performed the scoring, the data analysis and wrote the paper. P. Hollander planned the study, performed the scoring, and wrote the paper. R-M. Amini, G. Enblad, designed the study guided and reviewed the research and manuscript production. L. Löf performed the western blot and reviewed the manuscript. D. Molin, I. Glimelius, K. E. Smedby, H. Hjalgrim, J. Hashemi and G. Hedstrom reviewed and revised the primary data and the manuscript. All authors approved the manuscript and the interpretation of the data.

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REFERENCES

- Vardhana S, Younes A. The immune microenvironment in Hodgkin lymphoma: T cells, B cells, and immune checkpoints. *Haematologica*. 2016;101:794–802.
- Aldinucci D, Gloghini A, Pinto A, De Filippi R, Carbone A. The classical Hodgkin's lymphoma microenvironment and its role in promoting tumour growth and immune escape. *J Pathol*. 2010;221:248–63.
- Hollander P, Rostgaard K, Smedby KE, Molin D, Loskog A, de Nully Brown P, *et al.* An anergic immune signature in the tumor microenvironment of classical Hodgkin lymphoma is associated with inferior outcome. *Eur J Haematol*. 2018;100:88–97.
- Evens AM, Helenowski I, Ramsdale E, *et al.* A retrospective multicenter analysis of elderly Hodgkin lymphoma: outcomes and prognostic factors in the modern era. *Blood*. 2012;119:692–5.
- Glimelius I, Diepstra A. Novel treatment concepts in Hodgkin lymphoma. *J Intern Med*. 2017;281:247–60.
- Ansell SM. Immunotherapy in Hodgkin lymphoma: the road ahead. *Trends Immunol*. 2019;40:380–6.
- Chen R, Zinzani PL, Fanale MA, Armand P, Johnson NA, Brice P, *et al.* Phase II study of the efficacy and safety of pembrolizumab for relapsed/refractory classic Hodgkin lymphoma. *J Clin Oncol*. 2017;35:2125–32.
- Younes A, Santoro A, Shipp M, Zinzani PL, Timmerman JM, Ansell S, *et al.* Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol*. 2016;17:1283–94.

9. Ansell SM, Lesokhin AM, Borrello I, Mukundan S, Roemer MGM, Chapuy B, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2015;372:311–9.
10. Armand P, Shipp MA, Ribrag V, et al. Programmed death-1 blockade with pembrolizumab in patients with classical Hodgkin lymphoma after brentuximab Vedotin failure. *J Clin Oncol*. 2016;34:3733–9.
11. Veldman J, Visser L, van den Berg A, Diepstra A. Primary and acquired resistance mechanisms to immune checkpoint inhibition in Hodgkin lymphoma. *Cancer Treat Rev*. 2020;82:101931.
12. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD Jr, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell*. 2009;138:286–99.
13. Willingham SB, Volkmer J-P, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPα) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A*. 2012;109:6662–7.
14. Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, et al. Anti-CD47 antibody synergises with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142:699–713.
15. Patel K, Ramchandren R, Maris M, Lesokhin AM, von Keudell GR, Cheson BD, et al. Investigational CD47-blocker TTI-622 shows single-agent activity in patients with advanced relapsed or refractory lymphoma: update from the ongoing first-in-human dose escalation study. *Blood*. 2020;136:46–7.
16. Horwitz SM, Foran JM, Maris M, Lue JK, Sawas A, Okada C, et al. Updates from ongoing, first-in-human phase I dose escalation and expansion study of TTI-621, a novel biologic targeting CD47, in patients with relapsed or refractory hematologic malignancies. *Blood*. 2020;136:41–3.
17. Advani R, Flinn I, Popplewell L, Forero A, Bartlett NL, Ghosh N, et al. CD47 blockade by Hu5F9-G4 and rituximab in non-Hodgkin's lymphoma. *N Engl J Med*. 2018;379:1711–21.
18. López-Pereira B, Fernández-Velasco AA, Fernández-Vega I, Cortes-Torres D, Quirós C, Villegas JA, et al. Expression of CD47 antigen in reed-Sternberg cells as a new potential biomarker for classical Hodgkin lymphoma. *Clin Transl Oncol*. 2020;22:782–5.
19. Smedby KE, Hjalgrim H, Melbye M, et al. Ultraviolet radiation exposure and risk of malignant lymphomas. *J Natl Cancer Inst*. 2005;97:199–209.
20. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127:2375–90.
21. Carbone PP, Kaplan HS, Musshoff K, et al. Report of the committee on Hodgkin's disease staging classification. *Cancer Res*. 1971;31:1860–1.
22. Gobbi PG, Cavalli C, Gendarini A, et al. Reevaluation of prognostic significance of symptoms in Hodgkin's disease. *Cancer*. 1985;56:2874–80.
23. Hjalgrim H, Smedby KE, Rostgaard K, et al. Infectious mononucleosis, childhood social environment, and risk of Hodgkin lymphoma. *Cancer Res*. 2007;67:2382–8.
24. Hollander P, Kamper P, Smedby KE, et al. High proportions of PD-1+ and PD-L1+ leukocytes in classical Hodgkin lymphoma microenvironment are associated with inferior outcome. *Blood Adv*. 2017;1:1427–39.
25. Gholiha AR, Hollander P, Hedstrom G, et al. High tumour plasma cell infiltration reflects an important microenvironmental component in classic Hodgkin lymphoma linked to presence of B-symptoms. *Br J Haematol*. 2019;184:192–201.
26. Glimelius I, Rubin J, Rostgaard K, et al. Predictors of histology, tissue eosinophilia and mast cell infiltration in Hodgkin's lymphoma—a population-based study. *Eur J Haematol*. 2011;87:208–16.
27. Kampf C, Olsson I, Ryberg U, et al. Production of tissue microarrays, immunohistochemistry staining and digitalization within the human protein atlas. *J Vis Exp*. 2012;(63):3620.
28. Glimelius I, Qvarnström F, Simonsson M, et al. Tissue microarray and digital image analysis: a methodological study with special reference to the microenvironment in Hodgkin lymphoma. *Histopathology*. 2012;61:26–32.
29. Fedchenko N, Reifemuth J. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review. *Diagn Pathol*. 2014;9:221–1.
30. Austin PC. The use of propensity score methods with survival or time-to-event outcomes: reporting measures of effect similar to those used in randomised experiments. *Stat Med*. 2014;33:1242–58.
31. Lagerlöf I, Holte H, Glimelius I, et al. No excess long-term mortality in stage I-IIA Hodgkin lymphoma patients treated with ABVD and limited field radiotherapy. *Br J Haematol* Epub ahead of print. 2019;14:685–691.
32. Chao MP, Tang C, Pachynski RK, et al. Extranodal dissemination of non-Hodgkin lymphoma requires CD47 and is inhibited by anti-CD47 antibody therapy. *Blood*. 2011;118:4890–901.
33. Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol*. 2001;11:130–5.
34. Sick E, Boukhari A, Deramaudt T, et al. Activation of CD47 receptors causes proliferation of human astrocytoma but not normal astrocytes via an Akt-dependent pathway. *Glia*. 2011;59:308–19.
35. Huang C-Y, Ye Z-H, Huang M-Y, et al. Regulation of CD47 expression in cancer cells. *Transl Oncol*. 2020;13:100862.
36. Yang K, Xu J, Liu Q, et al. Expression and significance of CD47, PD1 and PDL1 in T-cell acute lymphoblastic lymphoma/leukemia. *Pathol Res Pract*. 2019;215:265–71.
37. Barclay AN, Van den Berg TK. The interaction between signal regulatory protein alpha (SIRPα) and CD47: structure, function, and therapeutic target. *Annu Rev Immunol*. 2014;32:25–50.
38. de Almeida AC, Barbosa SM, de Lourdes Rios Barjas-Castro M, et al. IFN-β, IFN-γ, and TNF-α decrease erythrophagocytosis by human monocytes independent of SIRP-α or SHP-1 expression. *Immunopharmacol Immunotoxicol*. 2012;34:1054–9.
39. Wang X, Yang L, Huang F, et al. Inflammatory cytokines IL-17 and TNF-α up-regulate PD-L1 expression in human prostate and colon cancer cells. *Immunol Lett*. 2017;184:7–14.
40. Green MR, Rodig S, Juszczynski P, et al. Constitutive AP-1 activity and EBV infection induce PD-L1 in Hodgkin lymphomas and post-transplant lymphoproliferative disorders: implications for targeted therapy. *Clin Cancer Res*. 2012;18:1611–8.
41. Anastasiadou E, Stroopinsky D, Alimperti S, et al. Epstein-Barr virus-encoded EBNA2 alters immune checkpoint PD-L1 expression by down-regulating miR-34a in B-cell lymphomas. *Leukemia*. 2019;33:132–47.
42. Nakayama A, Abe H, Kunita A, et al. Viral loads correlate with upregulation of PD-L1 and worse patient prognosis in Epstein-Barr virus-associated gastric carcinoma. *PLoS One*. 2019;14:e0211358.
43. Biondi-Zoccai G, Romagnoli E, Agostoni P, et al. Are propensity scores really superior to standard multivariable analysis? *Contemp Clin Trials*. 2011;32:731–40.
44. Mangano C, Ferrarini A, Forcato C, et al. Precise detection of genomic imbalances at single-cell resolution reveals intra-patient heterogeneity in Hodgkin's lymphoma. *Blood Cancer J*. 2019;9:92.
45. Wienand K, Chapuy B, Stewart C, et al. Genomic analyses of flow-sorted Hodgkin reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood Adv*. 2019;3:4065–80.
46. Eichenauer DA, Engert A, Dreyling M, et al. Hodgkin's lymphoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2011;22 Suppl 6:vi55–58.

SUPPORTING INFORMATION

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How to cite this article: Gholiha AR, Hollander P, Löf L, Glimelius I, Hedstrom G, Molin D. Checkpoint CD47 expression in classical Hodgkin lymphoma. *Br J Haematol*. 2022;197:580–589. <https://doi.org/10.1111/bjh.18137>