Expression of FOXP3, CD68, and CD20 at Diagnosis in the Microenvironment of Classical Hodgkin Lymphoma Is Predictive of Outcome

Paul Greaves, Andrew Clear, Rita Coutinho, Andrew Wilson, Janet Matthews, Andrew Owen, Milensu Shanyinde, T. Andrew Lister, Maria Calaminici, and John G. Gribben

Paul Greaves, Andrew Clear, Rita Coutinho, Andrew Wilson, Janet Matthews, Andrew Owen, T. Andrew Lister, Maria Calaminici, and John G. Gribben, Barts Cancer Institute, Queen Mary University of London, London; and Milensu Shanyinde, Centre for Statistics in Medicine, University of Oxford, Wolfson College, Oxford, United Kingdom.

Published online ahead of print at www.jco.org on October 8, 2012.

Supported by Cancer Research United Kingdom and The Baker Foundation.

Presented in part as an oral presentation at the 52nd Annual Meeting of the American Society of Hematology, Orlando, FL, December 4-7, 2010, and at the 11th International Conference on Malignant Lymphoma, Lugano, Switzerland, June 15-18, 2011.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Paul Greaves, MD, PhD, Centre for Haematology, Barts Cancer Institute, Queen Mary University of London, Charterhouse Square, London, United Kingdom EC1M 6BQ; e-mail: p.greaves@qmul.ac.uk.

© 2012 by American Society of Clinical Oncology

0732-183X/13/3102-256/\$20.00

DOI: 10.1200/JCO.2011.39.9881

ABSTRACT

Purpose

The immune microenvironment is key to the pathophysiology of classical Hodgkin lymphoma (CHL). Twenty percent of patients experience failure of their initial treatment, and others receive excessively toxic treatment. Prognostic scores and biomarkers have yet to influence outcomes significantly. Previous biomarker studies have been limited by the extent of tissue analyzed, statistical inconsistencies, and failure to validate findings. We aimed to overcome these limitations by validating recently identified microenvironment biomarkers (CD68, FOXP3, and CD20) in a new patient cohort with a greater extent of tissue and by using rigorous statistical methodology.

Patients and Methods

Diagnostic tissue from 122 patients with CHL was microarrayed and stained, and positive cells were counted across 10 to 20 high-powered fields per patient by using an automated system. Two statistical analyses were performed: a categorical analysis with test/validation set-defined cut points and Kaplan-Meier estimated outcome measures of 5-year overall survival (OS), disease-specific survival (DSS), and freedom from first-line treatment failure (FFTF) and an independent multivariate analysis of absolute uncategorized counts.

Results

Increased CD20 expression confers superior OS. Increased FOXP3 expression confers superior OS, and increased CD68 confers inferior FFTF and OS. FOXP3 varies independently of CD68 expression and retains significance when analyzed as a continuous variable in multivariate analysis. A simple score combining FOXP3 and CD68 discriminates three groups: FFTF 93%, 62%, and 47% ($P < .001$), DSS 93%, 82%, and 63% ($P = .03$), and OS 93%, 82%, and 59% ($P = .002$).

Conclusion

We have independently validated CD68, FOXP3, and CD20 as prognostic biomarkers in CHL, and we demonstrate, to the best of our knowledge for the first time, that combining FOXP3 and CD68 may further improve prognostic stratification.

J Clin Oncol 31:256-262. © 2012 by American Society of Clinical Oncology

INTRODUCTION

Classical Hodgkin lymphoma (CHL) is unique among the lymphomas in that the bulk of the infiltrate comprises not the malignant cells (Hodgkin and Reed-Sternberg cells) but inflammatory cells, including macrophages, T cells, B cells, neutrophils, eosinophils, fibroblasts, plasma cells, and mast cells.¹ The disease carries an excellent prognosis for most patients, with long-term remission greater than 80% following conventional chemotherapy or radiotherapy-based protocols.²⁻⁴ However, there remains a subset of patients whose disease is refractory to all conventional therapy,^{5,6} and many long-term survivors suffer the late effects of excessively toxic

treatments.⁷ Established clinical prognostic indices for advanced-stage disease, such as the initial prognostic score (IPS) from the International Prognostic Factors Project⁸ are rarely used to modify treatment. Risk-adapted therapy would improve management of this disease, but this requires identification of reliable biomarkers for favorable- and unfavorable-risk patients. Such biomarkers could also provide insight into the molecular biology of the disease.

The malignant cell is probably at least as dependent on extracellular signals as from endogenous signals arising from its own mutated genome.⁹ The macrophage appears to play a major role in tumor support,¹⁰ and previous studies suggested an adverse effect of increased macrophage infiltration.¹¹⁻¹⁴ The

tumor-dominating CD4⁺ T cells are also important in pathophysiology, but functional data are lacking.¹ Studies of solid tumor immune infiltrates have found an association between adverse outcome and increased expression of FOXP3,¹⁵ the marker of regulatory T cells (Tregs), a subset responsible for suppression of aberrant immune responses. In vivo Treg depletion leads to tumor regression,¹⁶ suggesting that the presence of Tregs suppresses effective immune response to tumor.^{17,18} However, in follicular lymphoma and CHL, the converse is found, with increased infiltrate associated with improved outcome.¹⁹⁻²⁶ The role of bystander B cells and other T-cell subsets is more controversial, although there is evidence suggesting that microenvironmental markers of B-cell function are associated with favorable outcome.²⁴ The role of Epstein Barr virus (EBV) is poorly understood despite a clear association with this disease. EBV is present in the malignant cell in 20% to 30% of cases, associated with the mixed-cellularity subtype and disease presenting in certain ethnic and age groups.²⁷ Immunohistochemistry (IHC) and gene-expression profiling (GEP) data suggest that EBV may influence the microenvironment.^{24,27}

Translation of studies based on tissue microarray/immunohistochemistry (TMA/IHC) to the clinical setting has been limited by experimental reproducibility and validity. This includes failure to apply consistent methodology, limited cohort sizes, inappropriate statistical methods (particularly optimal cut point analysis), failure to validate findings in independent patient cohorts, and the intrinsic fallibility of even expert histopathologists to count large numbers of cells across areas sufficiently large to draw conclusions regarding a heterogeneous microenvironment. Categorization of infiltrate extent as high, intermediate, or low leads to substantial data loss and interobserver discordance. Image analysis software can overcome some of these problems by counting huge numbers of events across larger regions, but it has its own limitations.

This study set out to examine the immune microenvironment in CHL and, in particular, to validate three CHL microenvironment-expressed biomarkers with demonstrated prognostic significance in recent studies: the macrophage marker CD68,¹² the regulatory T-cell marker FOXP3,²²⁻²⁵ and the B-cell marker CD20.²⁴ CD3, CD4, and CD8—nonspecific T, T-helper, and cytotoxic T-cell markers—were also assessed. Ten to 20 high-powered field (HPF) equivalents (2 to 3 mm²) were assessed by using image analysis software and were reported as continuous variables. This approach addresses major limitations of previous IHC work, namely limited regions of tumor assessment, categorization data loss, and interobserver variability.

PATIENTS AND METHODS

TMA/IHC and Image Analysis

We identified 122 adult patients of known clinical outcome, diagnosed at St. Bartholomew's Hospital, who had high-quality formalin-fixed paraffin-embedded tissue from the original diagnostic biopsy available. For the final analysis using all three biomarkers (CD20, CD68, and FOXP3), data from only 90 patients were available as a result of tissue loss and technical difficulties; however, this final cohort had characteristics similar to those of the original 122 patients. Characteristics of these patients are summarized in Table 1. Median follow-up was 16.5 years (range, 2 to 40 years). Triplicate 1-mm² cores were taken from regions of biopsy material identified by an expert histopathologist (M.C.) on sections stained with hematoxylin and eosin as being of high cellularity, containing malignant cells and avoiding fibrotic or acellular por-

Table 1. Patient Characteristics

Characteristic	No. of Patients in Original Cohort (N = 122)	% of Original Cohort	% of Patients Analyzed for All Three Biomarkers (n = 90)
Male	79	65	66
Age > 45 years	27	22	20
Advanced stage (IIB-IV)	87	71	71
Anthracycline-based chemotherapy	56	46	42
Alkylator-based chemotherapy	52	43	46
Radiotherapy only	14	11	12
Combined modality	48	39	34
Histologic subtype			
Nodular sclerosis	93	78	71
Mixed cellularity	25	20	27
Classical lymphocyte rich	0	—	—
Lymphocyte deplete	2	2	2
EBV+ (EBER-ISH)	38	31	36

NOTE. Right-most column indicates characteristics of final patient cohort of 90 for which data derived from all three biomarkers (FOXP3, CD68, and CD20) were available, which was not significantly different from the original cohort in terms of patient characteristics.

Abbreviations: EBV, Epstein-Barr virus; EBER-ISH, EBV-encoded RNA in situ hybridization.

tions. Sample collection followed informed, written consent in accordance with the Declaration of Helsinki. Ethical approval for this study was obtained from the local regional ethics board. Cores were arrayed into a recipient paraffin block, sectioned, and transferred onto glass slides. Staining for CD3, CD4, CD8, CD68, and FOXP3 following dewaxing and pressure-cooker antigen retrieval²⁸ was performed by using primary antibodies and dilutions shown in Table 2. To discriminate intact lymphoid follicle resident CD20⁺ cells from those in the malignant microenvironment (nonfollicular CD20⁺ cells), dual-color staining was used, costaining for CD21, a follicular dendritic cell-specific marker (see Data Supplement 1 [online only] for detailed protocol). The presence of EBV in malignant cells was confirmed by using in situ hybridization.

The Ariol imaging system (Genetix, San Jose, CA) was used to quantify antibody staining of the TMAs as previously described.²⁹ To standardize tumor area cellularity and enable valid comparisons, all cores were reviewed manually, and the cell count was accepted only if the core was intact, with less than 10% total fibrotic or acellular area (Data Supplement 2 Fig 2, online only). Each case was entered into the subsequent analysis only if there were two or three high-quality cores representing a total of 2 to 3 mm² or 10 to 20 HPFs. A mean count per 1-mm² core for lymphoid markers and mean percentage area for CD68 were then calculated. From the original cohort, there was further

Table 2. Primary Antibodies Showing Clone and Supplier, With Optimized Dilution Used for This Study

Antigen	Clone	Dilution	Supplier
CD3	SP7	1/500	Lab Vision
CD4	368	1/500	Novocastra
CD8	C8/144B	1/400	Dako
CD20	L26	1/2,000	Dako
CD21	2G9	1/130	Novocastra
CD68	KP1	1/8,000	Dako
FOXP3	263A/E7	1/100	Abcam

attrition of patients on the basis of these quality criteria, but for all markers, the total number of patients available was 90 or greater.

Statistical Analysis

To increase the robustness of statistical inferences, two independent analyses were performed: categorical and continuous data analysis. The first was a clinically applicable method, dividing patients categorically according to expression of each biomarker into cohorts on the basis of levels of expression (high and low or high, intermediate, and low). The continuous data analysis analyzed the prognostic effect of each of these biomarkers treated as continuous variables by using Cox regression analysis. Outcomes, measured from date of diagnosis to occurrence of event or date of last follow-up, were overall survival (OS), the event being death as a result of any cause, disease-specific survival (DSS), the event being death as a result of disease or complications of treatment for active disease, and freedom from first-line treatment failure (FFTF), the event being first relapse or progression of disease on first-line therapy, or therapy changed as a result of refractory disease.

Categorical (cut point) Data Analysis

Data analysis was performed by using the X-Tile statistical package³⁰ (Yale University, New Haven, CT) enabling cut points to be determined for markers without validated normal ranges. X-Tile divides the cohort into two independent data sets—a test set and a validation set—in a 1:2 ratio, determines optimal cut points for each marker for the test set, and applies this to the validation set.³¹ Kaplan-Meier curves defined by these cut points were generated, and statistical significance of differences arising from differential expression of each marker were determined by using the log-rank test.

Continuous Data Analysis

Cox proportional hazards models were used for this analysis to obtain estimates of hazard ratios along with 95% CIs for CD20, FOXP3, and CD68 and for the clinical risk factors for each outcome. The aim was to develop a predictive prognostic score incorporating age for all patients and for patients with advanced-stage disease; age, presence of stage IV disease at diagnosis, along with the clinical risk score (IPS) derived from the International Prognostic Factors Project⁸ (presence of > three risk factors considered to represent

high-risk disease). There is no universally accepted prognostic score for early-stage disease, and there is insufficient clinical information available to establish one for this cohort. Full details on statistical methodology are available in Data Supplement 1.

RESULTS

Categorical Data Analysis

Heterogeneity of expression of all biomarkers between patients is depicted in Data Supplement 1 Table 1 and Data Supplement 2 Figure 3 (online only). None of the biomarkers analyzed were found to be differentially expressed according to EBV status or histologic subtype (Data Supplement 2 Fig 8, online only).

Prognostic Significance of CD68 Expression

Three prognostic groups with low, intermediate, and high CD68 density were defined with cut points of less than 5%, 5% to 15%, and more than 15% by using the X-Tile software (Fig 1), the favorable group having the lowest CD68⁺ density. OS was significantly different for low, intermediate, and high groups ($P = .02$) with 5-year OS of 89%, 80%, and 65%, respectively. FFTF was also significantly different ($P = .001$). The prognostic significance for FFTF was maintained in subgroups presenting with advanced (73%, 63%, and 33%; $P = .03$) and early-stage disease (92%, 70%, and 20%; $P = .01$).

Prognostic Significance of FOXP3 Expression

No significant association of overall CD3⁺, CD4⁺, or CD8⁺ cell infiltrate on prognosis was found. However we confirm that FOXP3 cell density discriminated prognostic groups. By using X-Tile-defined

Fig 1. CD68 expression and outcome. Representative examples of (A) low and (B) high expression of CD68 in classical Hodgkin lymphoma (magnification ×20). CD68 expression is shown by horseradish peroxidase–diaminobenzidine (HRP-DAB) immunostaining. Time to (C) first-line treatment failure and (D) overall survival (OS) of patients based on area of low (< 5%), intermediate (INT; 5% to 15%) or high (> 15%) CD68 expression. FFTF, freedom from first-line treatment failure.

Fig 2. FOXP3 expression and outcome. Representative examples of (A) low and (B) high FOXP3 expression in classical Hodgkin lymphoma (magnification $\times 20$). FOXP3 expression is shown by horseradish peroxidase–diaminobenzidine (HRP–DAB) immunostaining. Time to (C) first-line treatment failure and (D) overall survival (OS) of patients on the basis of low (< 125 /hpf), intermediate (125–500/hpf) or high (> 500 hpf) numbers of FOXP3 expressing cells. FFTF, freedom from first-line treatment failure.

cut points of less than 125, 125 to 500, and more than 500 nuclei/HPF, the favorable prognosis group had the highest FOXP3⁺ density (Fig 2). OS ($P = .002$) and FFTF ($P = .006$) were significantly different for low, intermediate, and high groups, with 5-year OS of 68%, 80%, and 94%, respectively. The prognostic significance was maintained for both advanced (FFTF: 48%, 60%, and 72%; $P = .04$) and early-stage disease (FFTF: 57%, 67%, and 100%, $P = .04$).

Prognostic Significance of CD20 Expression

No prognostic significance was found for total CD20 expression. Nonfollicular CD20 expression, however, was found to influence survival. By using the X-Tile software, a cut point of 1,700 cells/mm² (250 cells/HPF) defined two prognostic groups with low and high nonfollicular CD20⁺ cell density (Fig 3). OS was improved for patients with high nonfollicular CD20 expression ($P = .003$), 87% versus 70% at 5 years, 84% versus 52% at 10 years, and 76% versus 43% at 20 years (Fig 3C). There was no significant difference between the groups by FFTF (64% v 57%; $P = .27$) or DSS (90% v 74%; $P = .09$).

Combined Prognostic Score Incorporating CD68 and FOXP3

We next sought to determine whether expression of CD68, FOXP3, and nonfollicular CD20 were related or independent variables. There was significant correlation between expression of nonfollicular CD20 and FOXP3 (data not shown), although there was no significant correlation between FOXP3 and CD68 ($P = .684$; Appendix Fig A1, online only). We next determined whether a combined score of these two markers provided additional prognostic information. Suitable cores were available for both FOXP3 and CD68 from 98 patients. Patients were allocated into a good-, intermediate-, or poor-

risk combined FOXP3/CD68 score (FOX/Mac) according to the risk group for each marker individually, as described in Appendix Figure A2 (online only). Outcomes based on categorization by FOX/Mac for good, intermediate, and poor risk were significantly different for FFTF ($P < .001$), with 5-year FFTF of 93%, 62%, and 47%; DSS ($P = .03$), with 5-year DSS of 93%, 82%, and 63% (data not shown); and OS ($P = .002$), with 5-year OS of 93%, 82%, and 59%, respectively (Figs 4A and 4B). The statistical significance was maintained for both early- and advanced-stage patients, with 5-year FFTF for early-stage patients of 100%, 72%, and 25% ($P = .005$) and advanced-stage patients of 92%, 62%, and 50% ($P = .008$; Figs 4C and 4D).

Continuous Data Analysis

The distribution of FOXP3, CD68, and CD20 was assessed by using dot plot graphs, and a transformation of each of the three markers using the log function was used to facilitate multivariate analysis (Data Supplement 2 Fig 3, online only). The univariate and multivariate analyses of the continuous data are summarized in Data Supplement 1 Tables 4 to 6 (online only).

OS

In univariate analysis that incorporated the biomarkers as continuous variables, only age, CD68, and FOXP3 were significant prognostic factors for OS. Multivariate analysis using a Cox proportional hazards model (based on 113 patients with available data and 37 observed deaths) included age and FOXP3. In this model, there was no significant overall interaction term effect determined between FOXP3 and CD68 ($P = .475$). There was a significantly greater risk of mortality associated with older age and lower levels of FOXP3. The repeated multivariate analysis that included clinical factors⁸ in the model was

Fig 3. CD20 expression and outcome. Representative examples of (A) low and (B) high nonfollicular expression of CD20 in classical Hodgkin lymphoma (magnification $\times 20$). CD20 expression is shown by horseradish peroxidase–diaminobenzidine (HRP-DAB) immunostaining. (C) Overall survival of patients based on low (< 250 cells/hpf [high-powered field]) or high (≥ 250 cells/hpf) CD20-expressing cells.

based on 76 advanced-stage patients and 28 deaths. Only FOXP3 was an independent prognostic variable ($P = .005$). The model that best explained data variation included age and FOXP3 (prognostic index [PI] = $0.040 \times [\text{age}] - 0.00017 \times [\text{FOXP3 absolute}]$) in which Harrell's c-index was 0.709, indicating that both covariates in the model explain the variation in OS reasonably well (Data Supplement 1 Table 7 and Data Supplement 2 Fig 4, online only).

DSS

In univariate analysis, only age and FOXP3 were significant prognostic factors for DSS. Multivariate analysis (based on 113 patients with available data and 24 observed events) included age and FOXP3. There was no significant interaction between FOXP3 and CD68 ($P = .423$). The repeated multivariate analysis that included clinical factors was based on 76 advanced-stage patients and 20 events. Only FOXP3 was an independent prognostic variable ($P = .015$), and the model that best explained variation in the data included age and FOXP3 (PI = $0.0293 \times [\text{age}] - 0.00028 \times [\text{FOXP3 absolute}]$) in which Harrell's c-index was 0.719 (Data Supplement 1 Table 7 and Data Supplement 2 Fig 5).

FFTF

In univariate analysis, only stage, CD68, and FOXP3 were significant prognostic factors for FFTF. Multivariate analysis (based on 113 patients with available data and 50 observed events) included presence of stage IV disease and FOXP3. There was no significant overall interaction effect between FOXP3 and CD68 ($P = .728$). The repeated multivariate analysis using prognostic score was based on 76 advanced-stage patients and 35 events. Only FOXP3 was an independent prognostic variable ($P = .03$), and the model best explaining

variation in the data included FOXP3 and presence of stage IV disease (PI = $0.635n - 0.00021 \times [\text{FOXP3 absolute}]$ where $n = 1$ if the patient has Stage IV disease and $n = 0$ otherwise) in which Harrell's c-index was 0.656 (Data Supplement 1 Table 7 and Data Supplement 2 Fig 6, online only).

DISCUSSION

A biomarker signature that is validated robustly, is powerfully predictive, and is cost-effective for clinical implementation remains elusive in CHL. Few biomarkers have translated into clinical practice because of flawed statistical analyses and failure to validate findings. This study validates three recently proposed prognostic biomarkers in a new patient cohort and, to the best of our knowledge for the first time, incorporates an automated system of image analysis to generate continuous variables applied to a multivariate analysis, which eliminates the potential data loss of categorization of data and examines dose-effects of expression of each biomarker. This study includes a separate analysis of categorical data comparable to the studies it set out to validate and more applicable to manual scoring and clinical translation. For each of the CD68, FOXP3, and CD20 biomarkers, the prognostic association suggested in previous studies is confirmed.

Nonfollicular CD20 expression was predictive of superior OS in the categorical analysis without retaining a dose-effect in the continuous data analysis. High CD68 expression predicted adverse outcome by all survival measures in the categorical analysis but showed a dose-effect only for OS in the continuous variable analysis and was not significant in the multivariate analysis. High FOXP3 expression emerged as the most robust predictor of superior outcome in both independent statistical analyses. As a continuous variable, this marker

Fig 4. Kaplan-Meier survival analysis stratifying by a combined score incorporating both FOXP3 and CD68 expression (FOX/Mac). Derivation of FOX/Mac is shown in Appendix Figure A2 (online only) using cut points for FOXP3 expression shown in Figure 2 and CD68 shown in Figure 1. (A) Time to first-line treatment failure on the basis of FOX/Mac score for entire cohort. (B) Overall survival (OS) on the basis of FOX/Mac score for entire cohort. (C) Time to first-line treatment failure on the basis of FOX/Mac score for patients with early-stage disease only. (D) Time to first-line treatment failure for patients with advanced-stage disease only. FFTF, freedom from first-line treatment failure.

showed a dose-effect that was significant in both univariate and multivariate analyses. It retained predictive power as a categorized variable in a simplified scoring system applicable to manual scoring or clinical use (high, medium, and low infiltration). A combined categorical score incorporating both FOXP3 and CD68 was able to discriminate patients with particularly poor and good risk in both early-stage and advanced-stage disease. A prognostic index based on the absolute number of FOXP3 infiltrating cells and the patient's age as continuous variables was able to predict OS and DSS better than an index that incorporated clinical factors alone.

TMA stained for various biomarkers can be used to characterize the CHL microenvironment, better understand disease pathophysiology, and measure patient heterogeneity to predict outcome. Much of the published research on IHC is limited by experimental and statistical methodology and hence is inadequate for translation into clinical practice. Conclusions drawn from GEP of the microenvironment in CHL are limited by the heterogeneity of the tissue represented, and different groups have proposed quite different prognostic GEP patterns arising from different experimental and statistical methodologies.^{12,24,32} Large-scale validation or clinical application of these findings is made difficult by the expense of the technology and lack of consistent findings. Finding that a biomarker is predictive of prognosis is merely a description of correlation and suggests, but does not robustly confirm, a biologic mechanism of functional importance. For example, the benefit conferred by FOXP3 expression could indicate that Tregs directly suppress the malignant B cell in CHL or suppress tumor-supporting T cells in the microenvironment. Alternatively, it may be a surrogate marker for another aspect of tumor biology or host

immune response that is beneficial in the eradication of residual disease after treatment. It may also simply represent a nonspecific T-cell activation marker. The favorable impact of CD20 expression may indicate that normal B cells are contributing to antitumor response or represent malignant cell precursors that are more responsive to treatment. Our findings that CD20 expression confers an impact on OS but not on FFTF suggest a complex role for normal B cells in CHL, including interactions with host immunity, therapeutic late effects, late relapse, salvage, and overall host fitness. The role of the macrophage in tumor promotion has been demonstrated far more robustly in biologic models,^{10,11} but further functional work is required to demonstrate the pathophysiologic importance of individual biomarkers. The absence of representative *in vivo* or *in vitro* models of CHL remains problematic and is the focus of ongoing investigation.

Notwithstanding these limitations, correlative IHC biomarker data, generated appropriately, examined with statistical rigor, and validated extensively could contribute to clinical decision making in CHL. Functional imaging has shown considerable promise as a prognostic tool by demonstrating early treatment responsiveness,³³ but pretreatment prognostic biomarkers are scarce, and none are used routinely to guide treatment. This study has validated and confirmed recently proposed prognostic biomarkers by examining a greater extent of pretreatment lymph node–derived tissue per patient than any published work in CHL to date and by analyzing the use of test/validation methodology for cut point determination and independently as continuous variables in a multivariate analysis to demonstrate dose-responsiveness and avoid data loss from categorization of data. We propose that by using only the widely available IHC

markers CD68 and FOXP3, with cut points as described, a simple prognostic score can be developed that may be clinically translatable. To further validate these findings, we have initiated an international collaborative effort in two new independent patient cohorts and are using predefined cut points and both automated and manual counting methods across three different laboratories.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy,

please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory**

Role: None **Stock Ownership:** None **Honoraria:** John G. Gribben, Roche, Celgene, Mundipharma, Merck **Research Funding:** None **Expert**

Testimony: None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Paul Greaves, John G. Gribben

Collection and assembly of data: Paul Greaves, Andrew Clear, Andrew Wilson, Janet Matthews, Andrew Owen

Data analysis and interpretation: Paul Greaves, Andrew Clear, Rita Coutinho, Milensu Shanyinde, T. Andrew Lister, Maria Calaminici, John G. Gribben

Manuscript writing: All authors

Final approval of manuscript: All authors

REFERENCES

- Steidl C, Connors JM, Gascoyne RD: Molecular pathogenesis of Hodgkin's lymphoma: Increasing evidence of the importance of the microenvironment. *J Clin Oncol* 29:1812-1826, 2011
- Engert A, Diehl V, Franklin J, et al: Escalated-dose BEACOPP in the treatment of patients with advanced-stage Hodgkin's lymphoma: 10 years of follow-up of the GHSG HD9 study. *J Clin Oncol* 27:4548-4554, 2009
- Engert A, Plütschow A, Eich HT, et al: Reduced treatment intensity in patients with early-stage Hodgkin's lymphoma. *N Engl J Med* 363:640-652, 2010
- Viviani S, Zinzani PL, Rambaldi A, et al: ABVD versus BEACOPP for Hodgkin's lymphoma when high-dose salvage is planned. *N Engl J Med* 365:203-212, 2011
- Varterasian M, Ratanatharathorn V, Uberti JP, et al: Clinical course and outcome of patients with Hodgkin's disease who progress after autologous transplantation. *Leuk Lymphoma* 20:59-65, 1995
- Shamash J, Lee SM, Radford JA, et al: Patterns of relapse and subsequent management following high-dose chemotherapy with autologous haematopoietic support in relapsed or refractory Hodgkin's lymphoma: A two centre study. *Ann Oncol* 11:715-719, 2000
- Castellino SM, Geiger AM, Mertens AC, et al: Morbidity and mortality in long-term survivors of Hodgkin lymphoma: A report from the Childhood Cancer Survivor Study. *Blood* 117:1806-1816, 2011
- Hasenclever D, Diehl V: A prognostic score for advanced Hodgkin's disease: International Prognostic Factors Project on Advanced Hodgkin's Disease. *N Engl J Med* 339:1506-1514, 1998
- Küppers R: The biology of Hodgkin's lymphoma. *Nat Rev Cancer* 9:15-27, 2009
- Condeelis J, Pollard JW: Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124:263-266, 2006
- Normann SJ: Macrophage infiltration and tumor progression. *Cancer Metastasis Rev* 4:277-291, 1985
- Steidl C, Lee T, Shah SP, et al: Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* 362:875-885, 2010
- Tzankov A, Matter MS, Dirnhofer S: Refined prognostic role of CD68-positive tumor macrophages in the context of the cellular microenvironment of classical Hodgkin lymphoma. *Pathobiology* 77:301-308, 2010
- Kamper P, Bendix K, Hamilton-Dutoit S, et al: Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. *Haematologica* 96:269-276, 2011
- Curiel TJ, Coukos G, Zou L, et al: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942-949, 2004
- Zou W: Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295-307, 2006
- Woo EY, Chu CS, Goletz TJ, et al: Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61:4766-4772, 2001
- Woo EY, Yeh H, Chu CS, et al: Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 168:4272-4276, 2002
- Lee AM, Clear AJ, Calaminici M, et al: Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. *J Clin Oncol* 24:5052-5059, 2006
- Yang ZZ, Novak AJ, Stenson MJ, et al: Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma. *Blood* 107:3639-3646, 2006
- Carreras J, Lopez-Guillermo A, Fox BC, et al: High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* 108:2957-2964, 2006
- Alvaro T, Lejeune M, Salvadó MT, et al: Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clin Cancer Res* 11:1467-1473, 2005
- Kelley TW, Pohlman B, Elson P, et al: The ratio of FOXP3+ regulatory T cells to granzyme B+ cytotoxic T/NK cells predicts prognosis in classical Hodgkin lymphoma and is independent of bcl-2 and MAL expression. *Am J Clin Pathol* 128:958-965, 2007
- Chetaille B, Bertucci F, Finetti P, et al: Molecular profiling of classical Hodgkin lymphoma tissues uncovers variations in the tumor microenvironment and correlations with EBV infection and outcome. *Blood* 113:2765-2775, 2009
- Tzankov A, Meier C, Hirschmann P, et al: Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. *Haematologica* 93:193-200, 2008
- Beyer M, Classen S, Endl E, et al: Comparative approach to define increased regulatory T cells in different cancer subtypes by combined assessment of CD127 and FOXP3. *Clin Dev Immunol* 2011:734036, 2011
- Flavell KJ, Murray PG: Hodgkin's disease and the Epstein-Barr virus. *Mol Pathol* 53:262-269, 2000
- Norton AJ, Jordan S, Yeomans P: Brief, high-temperature heat denaturation (pressure cooking): A simple and effective method of antigen retrieval for routinely processed tissues. *J Pathol* 173:371-379, 1994
- Kleiner HE, Krishnan P, Tubbs J, et al: Tissue microarray analysis of eIF4E and its downstream effector proteins in human breast cancer. *J Exp Clin Cancer Res* 28:5, 2009
- Camp RL, Dolled-Filhart M, Rimm DL: X-tile: A new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res* 10:7252-7259, 2004
- Altman DG, Lausen B, Sauerbrei W, et al: Dangers of using "optimal" cutpoints in the evaluation of prognostic factors. *J Natl Cancer Inst* 86:829-835, 1994
- Sánchez-Espiridión B, Montalbán C, López A, et al: A molecular risk score based on 4 functional pathways for advanced classical Hodgkin lymphoma. *Blood* 116:e12-e17, 2010
- Hutchings M, Loft A, Hansen M, et al: FDG-PET after two cycles of chemotherapy predicts treatment failure and progression-free survival in Hodgkin lymphoma. *Blood* 107:52-59, 2006