A Gene Expression–based Model to Predict Metabolic Response After Two Courses of ABVD in Hodgkin Lymphoma Patients

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ABSTRACT

Purpose: Early response to ABVD, assessed with interim FDG-PET (iPET), is prognostic for classical Hodgkin lymphoma (cHL) and supports the use of response adapted therapy. The aim of this study was to identify a gene-expression profile on diagnostic biopsy to predict iPET positivity (iPET+).

Experimental Design: Consecutive untreated patients with stage I–IV chL who underwent iPET after two cycles of ABVD were identified. Expression of 770 immune-related genes was analyzed by digital expression profiling (NanoString Technology). iPET was centrally reviewed according to the five-point Deauville scale (DS 1–5). An iPET+ predictive model was derived by multivariate regression analysis and assessed in a validation set identified using the same inclusion criteria.

Results: A training set of 121 and a validation set of 117 patients were identified, with 23 iPET+ cases in each group. Sixty-three (52.1%), 19 (15.7%), and 39 (32.2%) patients had stage I–II, III, and IV, respectively. Diagnostic biopsy of iPET+ cHLs showed transcriptional profile distinct from iPET−. Thirteen genes were stringently associated with iPET+. This signature comprises two functionally stromal-related nodes. Lymphocytes/monocytes ratio (LMR) was also associated to iPET+. In the training cohort a 5–gene/LMR integrated score predicted iPET+ [AUC, 0.88; 95% confidence interval (CI), 0.80–0.96]. The score achieved a 100% sensitivity to identify DS5 cases. Model performance was confirmed in the validation set (AUC, 0.68; 95% CI, 0.52–0.84). Finally, iPET score was higher in patients with event versus those without.

Conclusions: In cHL, iPET is associated with a genetic signature and can be predicted by applying an integrated gene-based model on the diagnostic biopsy.

Introduction

Classical Hodgkin lymphoma (cHL) is a relatively rare, highly curable neoplasm of the immune system that typically affects young adults (1). For many years, treatment of cHL has been based on the administration of a full course of doxorubicin, vinblastine, bleomycin, and dacarbazine (ABVD) or of the more intense bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP). The two treatment options permit achieving similar cure rates, with higher relapse rates to first line with ABVD, and with more severe early and late toxicity with BEACOPP (2). More recently the use of FDG-PET in cHL has shown that metabolic response during or after chemotherapy is highly predictive of the subsequent risk of progression and of death; this has prompted to the definition of response adapted therapy (3, 4). Treatment adaptation to interim metabolic response has been studied mainly in patients initially treated with ABVD regimen either in early or in advanced stage (3, 4) and has recently been shown to be useful also with BEACOPP chemotherapy (5–7). Response adapted therapy has contributed to reducing unnecessary toxicity while preserving treatment efficacy in early responding patients and to the early treatment intensification of patients at higher risk of treatment failure. Whether this approach is translating into a significant improvement of patient outcome in the long term remains unknown, but the use of iPET to adapt subsequent treatment is recommended by most of the available guidelines (8, 9).

While interim assessment of response with FDG-PET has been identified as a critical decisional point in the management of patients with cHL as it informs on refractoriness to chemotherapy, no study so far has been conducted to investigate the biological background of persistent metabolic uptake. Also, the identification of baseline clinical and biological features to predict interim metabolic response represents a meaningful research question. Through the identification of baseline features that might accurately predict interim response and chemorefractoriness, it would be possible to identify high-risk patients before treatment start and to plan intensified therapies upfront.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Clin Cancer Res 2020;26:373-83

doi: 10.1158/1078-0432.CCR-19-2356

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Translational Relevance

Classical Hodgkin lymphoma (cHL) is a nonlinear, open, and intrinsically dynamic system of interconnected and mutually dependent components. The construction of new strategies for a more precise and personalized assessment of risks in cHL requires greater integration between datasets from different levels of organization, including deep biological investigations, detailed imaging profiles, and clinical features. This work provides new evidence that gene-expression profiling on diagnostic biopsies may be used to anticipate early metabolic response in cHL and paves the way to similar studies addressing the same scientific question and methodology in different cHL setting (therapies other than ABVD, relapsed cases) and in other lymphoma subtypes. Also, the availability of a genetic signature that is strongly correlated to early metabolic response, but that is available at time of diagnosis, represents a good rationale to move to assess the efficacy of this new tool in prospective clinical trials.

Biological variation by means of gene-expression analysis has shown a consistent relationship to treatment response and survival in other subtypes of B-cell lymphomas, thereby leading to the definition of molecularly distinct subclasses of patients with different risk profiles and different clinical needs. The application of gene-based models that measure biological variability is rapidly translating into the management of patients with B-cell lymphoma for a more appropriate risk-based stratification and management (10).

On the basis of these assumptions, we performed a gene-expression profiling analysis in pretreatment biopsies from patients with ABVD-treated cHL, with the aim to evaluate the biological basis of iPET metabolic response and to identify a gene signature that may anticipate chemorefractoriness.

Materials and Methods

Study design and patients

The study design utilized data from a training cohort to generate an iPET predictive gene-expression–based model and tested its performance in an independent validation cohort. Consecutive patients with stage I–IV classic cHL who underwent iPET after two cycles of ABVD treated at the Hematology Unit of Arcispedale S. Maria Nuova-IRCCS Hospital of Reggio Emilia were used as the training set. Required inclusion criteria were as follows: availability of formalin-fixed, paraffin-embedded (FFPE) tumor diagnostic biopsy from the Pathology Unit of the Reggio Emilia hospital; availability of baseline and interim PET Dicom images for review; and signed informed consent. A validation set was identified from the Hematology Unit of Careggi Hospital of Firenze using the same criteria. Histologic sections of all samples were reviewed by three different pathologists (R. Valli, M. Zanelli, R. Santi). This study was conducted in accordance with Declaration of Helsinki and was approved by the Internal Review Board. Written consent was obtained from all leaving patients, still in follow-up at our institution, after full explanation of the purpose and nature of the study.

Gene-expression profiling

Total RNA was extracted by Maxwell RSC RNA FFPE kit (Promega) starting from 5 slides of 5-μm FFPE tissue. RNA quantity and quality were assessed by NanoDrop2000 (Thermo Fisher Scientific). For samples that reached the quality standards (A260/A280 ≥ 1.7 and A260/A230 ≥ 1.8), we evaluated the expression profile by NanoString using the PanCancer Immune Profiling Panel (NanoString Technologies) as described previously (11). This panel includes 770 genes from 24 different immune cell types, covering both the adaptive and innate immune response. Analysis of detected gene counts was performed by nSolver Analysis Software 3.0 (NanoString Technologies). First, samples were selected by checking imaging quality controls: percentage of fields of view (FOV) read (>75%), binding density (between 0.05 and 2.25), positive control linearity (>0.95), and positive control limit of detection (>2). For samples that passed imaging quality controls, raw gene counts were normalized on technical controls and housekeeping genes included in the panel.

Mean count of negative controls plus two SDs was subtracted from each gene count to eliminate negative background. Then, normalization on synthetic positive controls was conducted by multiplying the count of each gene for a correction factor. This factor was calculated for each sample as the ratio between the quadratic mean of positive controls counts and the mean of quadratic means in all samples. To perform CodeSet Content normalization on housekeeping genes, ten reference genes were selected among the forty available in the panel based on the lowest coefficients of variation (CV = ratio between mean counts and SDs across all samples). Finally, CodeSet Content normalization was performed by multiplying genes counts for a further correction factor calculated on reference genes as described for positive technical controls.

After completion of normalization processes, counts were log-transformed and a build ratio analysis was performed by comparing the expression profiles of iPET+ and iPET− samples. For each comparison, the P value (as one-tailed Student t-test) and the false discovery rate (FDR) obtained by the Benjamini–Yekutieli method were calculated. Finally, genes were ranked on the basis of the lowest coefficients of variation (CV = ratio between mean counts and SDs across all samples). Finally, CodeSet Content normalization was performed by multiplying genes counts for a further correction factor calculated on reference genes as described for positive technical controls.

By using R packages pROC and ROCR, we constructed the receiver operating characteristic (ROC) curve and calculated the area under the ROC curve (AUC) to evaluate the optimum score threshold and the relative specificity, sensitivity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of score prediction.
Gene Predictive Model for Interim PET in Hodgkin Lymphoma

Table 1. Association of iPET response with clinicopathologic variables in training (n = 120) and validation (n = 111) cohorts.

| Variables          | Status | Training cohort | Validation cohort |
|--------------------|--------|----------------|------------------|
|                    |        | Pos (n) | Neg (n) | P  | Adj | Pos (n) | Neg (n) | P  | Adj |
| Age                | <45    | 15 (65.2%) | 58 (59.8%) | 0.81 | 0.12 | 15 (65.2%) | 59 (67.0%) | >0.99 | 0.72 |
|                    | >45    | 8 (34.8%) | 39 (40.2%) |  &lt;0.001 | 0.95 | 8 (34.8%) | 29 (33.0%) | 0.21 | 0.14 |
| Sex                | Male   | 11 (47.8%) | 49 (50.5%) | >0.99 | 0.95 | 15 (65.2%) | 42 (47.7%) | 0.21 | 0.14 |
|                    | Female | 12 (52.2%) | 48 (49.5%) |  &lt;0.001 | 0.95 | 8 (34.8%) | 46 (52.3%) | 0.21 | 0.14 |
| Leukocytes (×10^3 cells/mm^3) | <15  | 17 (73.9%) | 87 (89.7%) | 0.03 | 0.25 | 17 (73.9%) | 78 (88.6%) | 0.08 | 0.23 |
|                    | >15    | 6 (26.1%) | 8 (8.2%) |  &lt;0.001 | 0.95 | 6 (26.1%) | 9 (10.2%) | 0.21 | 0.14 |
| Lymphotoles (×10^3 cells/mm^3) | <0.6 | 2 (8.7%) | 10 (10.3%) | >0.99 | 0.97 | 2 (8.7%) | 1 (1.1%) | 0.11 | 0.66 |
|                    | >0.6   | 21 (91.3%) | 85 (87.6%) |  &lt;0.001 | 0.95 | 21 (91.3%) | 85 (96.6%) | 0.21 | 0.14 |
| LMR                | ≤2.1   | 17 (73.9%) | 42 (43.3%) | 0.02 | 0.05 | 12 (52.2%) | 41 (46.6%) | 0.81 | 0.47 |
|                    | >2.1   | 6 (26.1%) | 53 (54.6%) |  &lt;0.001 | 0.95 | 11 (47.8%) | 45 (51.1%) | 0.005 | 0.05 |
| Hemoglobin, g/DL   | <10.5  | 2 (8.7%) | 16 (16.5%) | 0.52 | 0.15 | 6 (26.1%) | 4 (4.5%) | 0.005 | 0.05 |
|                    | >10.5  | 21 (91.3%) | 79 (81.4%) | 0.81 | 0.04 | 17 (73.9%) | 83 (94.3%) | 0.58 | 0.61 |
| LDH/ULN            | 45     | 5 (21.7%) | 20 (21.7%) |  &lt;0.001 | 0.95 | 7 (30.4%) | 22 (25.0%) | 0.81 | 0.47 |
|                    | 50     | 15 (65.2%) | 43 (43.3%) | 0.81 | 0.04 | 12 (52.2%) | 55 (62.5%) | 0.58 | 0.61 |
|                    | >50    | 17 (73.9%) | 46 (47.4%) | 0.81 | 0.04 | 8 (34.8%) | 24 (27.3%) | 0.58 | 0.61 |
| ESR (mm/hour)      | <50    | 2 (8.7%) | 46 (47.4%) | 0.03 | 0.21 | 13 (56.5%) | 50 (56.8%) | 0.61 | 0.47 |
|                    | >50    | 17 (73.9%) | 46 (47.4%) | 0.81 | 0.04 | 8 (34.8%) | 24 (27.3%) | 0.61 | 0.47 |
| Stage              | I-II   | 8 (34.8%) | 54 (55.7%) | 0.11 | 0.09 | 9 (39.1%) | 52 (59.1%) | 0.14 | 0.37 |
|                    | III-IV | 15 (65.2%) | 43 (44.3%) | 0.81 | 0.04 | 14 (60.9%) | 36 (40.9%) | 0.28 | 0.97 |
| Symptoms           | A      | 14 (60.9%) | 41 (42.9%) | >0.99 | 0.97 | 12 (52.2%) | 35 (40.9%) | 0.28 | 0.97 |
|                    | B      | 9 (39.1%) | 36 (37.1%) | 0.81 | 0.04 | 11 (47.8%) | 29 (33.0%) | 0.28 | 0.97 |
| Bulky              | No     | 19 (82.6%) | 82 (84.5%) | 0.76 | 0.54 | 13 (56.5%) | 73 (83.0%) | 0.01 | 0.09 |
|                    | Yes    | 4 (17.4%) | 15 (15.5%) | 0.76 | 0.54 | 10 (43.5%) | 14 (16.5%) | 0.01 | 0.09 |
| Risk group         | Early  | 8 (34.8%) | 50 (51.5%) | 0.22 | 0.09 | 1 (4.3%) | 15 (17.0%) | 0.57 | — |
|                    | Advanced | 15 (65.2%) | 47 (48.5%) | 0.22 | 0.09 | 2 (8.7%) | 11 (12.5%) | 0.22 | 0.09 |

Note: ESR, erythrocyte sedimentation rate; LDH/ULN, lactate dehydrogenase/upper limit normal; P_adj, adjusted for all clinical variables considered in the table.
*Not included in multivariate analysis for validation cohort because value was missing for 88 patients (78%). Numbers in bold indicate statistically significant P value.

Results

Clinical features of patients in the training and validation cohorts

A training cohort (n = 121) and a separate validation cohort (n = 117) of consecutive patients with cHL were selected (Supplementary Table S1). iPET results were available for 120 and 111 patients for training and validation cohorts, respectively. In each cohort, 23 iPET⁺ patients were identified. FFPE samples at diagnosis was available for 119 patients of the training set and for 117 of the validation set. Multivariate logistic analysis showed that only the lymphocytes/monocytes ratio (LMR) was significantly associated with iPET⁺ in the training cohort (P = 0.05; Table 1).

A 13-gene signature discriminates patients with iPET⁺ from iPET⁻ cHL

Gene-expression profile in a panel of 770 immune-related genes was analyzed by digital expression profiling. After quality check controls and data normalization, profiles from 106 samples were eligible for further analysis. Among these patients, 21 (19.8%) were iPET⁺, 84 (79.2%) were iPET⁻; 1 (0.9%) had no iPET images available for revision and was therefore excluded (Fig. 1A). Differential analysis between iPET⁺- and iPET⁻-associated GEP identified 241 (33%) significantly deregulated genes (P < 0.05; Fig. 1B and D). The majority of these genes (n = 171, 71%) were upregulated while 70 genes (29%) were repressed in iPET⁻ compared with iPET⁺ cHLs. Principal component analysis (PCA) clustered iPET⁺ and iPET⁻ samples in two separate groups (Fig. 1C). Gene Ontology (GO) analysis of the 241 genes highlighted enrichment of many cancer-relevant pathways, including immune response, inflammation, and cell migration (Supplementary Fig. S1).

To develop a stringent gene signature associated with iPET⁺, we further filtered the 241 differentially expressed genes applying absolute fold change (FC ≥ 2) and FDR (FDR < 0.1) restraining cutoffs. We identified a list of 13 genes whose expression was positively correlated with iPET⁺ (Fig. 1D and E; Table 2). Protein–protein interaction analysis identified two stromal-related nodes within the 13-gene signature (Fig. 2A). The first comprised microenvironment-related immune-modulatory factors including the chemotactic cytokines CXCL2, CXCL3, and CCL18, the myeloid cells receptor TREM1, and the proinflammatory gene SAA1. The second comprised genes involved in cell movement, wound healing, and blood vessels organization, including the matrix components PLAU, FN1, and SPP1 and the membrane matrix–interacting proteins ITG5A, CD9, LRP1, and THBS1. The proangiogenetic factor VEGFA bridges functional connections between the two nodes.

A gene-based predictive model anticipates iPET response at diagnosis

We built a predictive model that based on GEP could anticipate iPET⁺. First, we performed an expression correlation analysis between the 13 genes of the signature to define possible collinearity between genes. We identified ITG5A, CD9, and FN1 as strongly interdependent (correlation coefficient = 0.8; Fig. 2B). Because of this collinearity, CD9 and FN1 were excluded and ITG5A was maintained in further analyses as representative of this node, being the most strongly associated to iPET⁺ (Table 2). Next, multivariate logistic regression
Lesions of iPET$^+$ patients show distinct biological features. A, Outline of the study workflow for the development of the iPET predictive risk score. B, Volcano plot displaying differential expressed genes between iPET$^+$ and iPET$^-$/C0 lesions. Black dots represent genes significantly deregulated ($P < 0.05$) and dashed lines indicate absolute FC $\geq 2$. C, Principal component analysis (PCA) shows the variance between iPET$^+$ (black dots) and iPET$^-$ (gray dots) samples explained by the 241 genes differentially expressed. D, Summary of genes differentially expressed between iPET$^+$ and iPET$^-$/C0 patients, using progressive selection criteria. E, Boxplots representing the expression of the 13 genes associated with iPET$^+$ in the training cohort.
was applied to identify genes whose expression was independently associated with iPET (Table 2). Five genes (ITGA5, SAA1, CXCL2, SPP1, and TREM1) remained significantly associated. LMR, the only clinical variable that was initially found associated with the iPET status, was also included in the multivariate analysis and resulted independently associated with iPET results. A final model based on these variables was built to develop an iPET predictive score. ROC curve for iPET response demonstrated the high discriminatory accuracy of the model [AUC, 0.88; 95% confidence interval (CI), 0.80–0.96; Fig. 2C and D]. Application of the score to the training cohort consistently segregated iPET⁺ from iPET⁻ cHLs (Fig. 3A). Figure 3B illustrates the contribution of each gene to the score and the distribution of its expression within the 104 samples of the training cohort ranked by score values. We also reported the distribution of LMR and the iPET results. LMR negatively correlated with iPET. We used the iPET predictive score to stratify the training cohort into quartiles obtaining the following distribution of true iPET⁺ patients: Q1–76.2%, Q2–9.5%, Q3–1.0%, and Q4–0.8%. Figure 3C shows a higher performance in predicting iPET⁺ patients and confirm their association with iPET⁺. In contrast, no significant differences were observed in the expression of these genes in the iPET⁻ groups comparing the two cohorts (Supplementary Fig. S2C). ROC analysis obtained an AUC of 0.68 (0.52–0.84), specificity of 69%, sensitivity of 64%, and accuracy of 68% (Fig. 3D). Even if a slight decrease in model performance was observed, the box-plot distribution shows that the iPET predictive score is consistently higher in iPET⁺ than in iPET⁻ patients and confirms their association with iPET⁺.

Table 2. Association of iPET response with top ranking gene in training cohort and the significantly correlated clinical variable (LMR).

| Gene name | Gene bank accession | FC | P     | FDR  | Multivariate P value | Multivariate P value including LMR |
|-----------|---------------------|----|-------|------|----------------------|------------------------------------|
| VEGFA     | NM_001025366.1      | 2.02| 1.568E-07 | 0.0008 | 0.504               | 0.649                               |
| PLA1      | NM_002658.2         | 2.07| 4.850E-06 | 0.008  | 0.972               | 0.500                               |
| THBS1     | NM_003246.2         | 2.35| 1.04E-04  | 0.055  | 0.899               | 0.952                               |
| ITGA5     | NM_002205.2         | 2.67| 1.294E-04 | 0.062  | 0.034b              | 0.023b                              |
| SAA1      | NM_199161.1         | 5.80| 1.701E-04 | 0.063  | 0.39                | 0.072b                              |
| FNI⁺      | NM_012482.1         | 3.92| 1.553E-04 | 0.063  | —                   | —                                   |
| LRPI      | NM_003532.2         | 2.22| 2.053E-04 | 0.063  | 0.202               | 0.100                               |
| CXCL2     | NM_002089.3         | 2.90| 2.347E-04 | 0.065  | 0.019b              | 0.008b                              |
| CCL18     | NM_002988.2         | 3.45| 4.512E-04 | 0.082  | 0.793               | 0.665                               |
| SPP1      | NM_000582.2         | 3.35| 5.039E-04 | 0.082  | 0.094b              | 0.060b                              |
| CD9⁺      | NM_001769.2         | 2.34| 4.894E-04 | 0.082  | —                   | —                                   |
| CXCL3     | NM_002090.2         | 2.98| 6.138E-04 | 0.090  | 0.656               | 0.618                               |
| TREM1     | NM_018643.3         | 5.29| 7.439E-04 | 0.099  | 0.160               | 0.079b                              |
| LMR       | —                   | —  | —     | —     | —                   | 0.095b                              |

Note: FDR (false discovery rate) calculated by Benjamini–Yekutieli method. Abbreviation: FC, fold change.

*pGenes excluded from multivariate analyses on the basis of correlation matrix result (Fig. 2B).

*pGenes included in the final model.

Score validation in an independent cohort

Out of the 117 cHLs of the validation cohort, 89 yielded RNA suitable for GEP analysis, while 7 additional samples did not pass post-run quality check controls and data normalization and were therefore excluded (Fig. 4A). Of the remaining 82 cHLs, 14 were iPET⁺ (17.1%) and 68 were iPET⁻ (82.9%). Figure 4B reports expression trend of the 13-gene stromal signature in iPET⁺ and iPET⁻ patients and confirms their association with iPET⁺. In contrast, no significant differences were observed in the expression of these genes in the iPET⁻ groups comparing the two cohorts (Supplementary Fig. S3). ROC analysis obtained an AUC of 0.68 (0.52–0.84), specificity of 69%, sensitivity of 64%, and accuracy of 68% (Fig. 4C). Even if a slight decrease in model performance was observed, the box-plot distribution shows that the iPET predictive score is consistently higher in iPET⁺ than in iPET⁻ cHLs (P = 0.03; Fig. 4D) confirming the validity of the model.

I PET⁺ predictive score and treatment failure

We conducted an exploratory analysis with the aim of assessing the potential association of the iPET predictive score with TF within the entire cohort (training and validation). TF was defined as one of the following: change of therapy after iPET⁺ (TC), lack of metabolic response at final PET (PETD), PD, whichever came first. Only patients with at least 3 years of follow-up and for whom iPET predictive score was available were included (n = 115). TF was identified in 26 patients (22.6%) and included TC (n = 11), iPET⁺ (n = 12), and PD (n = 3). Boxplot distribution demonstrated that the iPET predictive score was significantly higher in patients with TF versus TF⁻ cHL (P = 0.02; Fig. 4E). Finally, we correlated the iPET score with TTF. The median follow-up of our series was 36 months (range 2–114), 3-year TTF was 79.6%, and no significant correlation was found (data not shown). Furthermore, patients with iPET predictive score above the threshold had increased rates of TF (31.2% vs. 19.1%; Supplementary Fig. S3A). We also investigated the iPET predictive score in patients that experienced clinical event according to PFS, without considering the iPET positivity (PFS⁺ vs. PFS⁻; Supplementary Fig. S3B). The trend was confirmed but the difference did not reach significance.
statistical significance ($P = 0.43$), likely due to the limited number of PFS$^+$ patients remaining in the cohort. Administered treatments in both training and validation sets are summarized in Supplementary Table S2.

Discussion

cHL is a relatively rare neoplastic disease of the immune system that mainly affects young adults. The achievement of high cure rates and
the young age of the patients have progressively shifted the interest of clinical research from survival improvement programs to personalized therapy programs with the aim of obtaining cure without treatment-induced side effects. The identification of prognostic factors able to predict with sufficient accuracy the individual risk of the patient and to adapt accordingly the intensity of the treatments is crucial and it is the base of personalized treatments.

Here we analyzed the GEP of a consecutive series of patients with cHL and we developed an early metabolic response predictor that identifies at diagnosis those patients with an increased probability of obtaining a positive iPET after 2 courses of ABVD. The model was tested in an independent patient cohort, for which it accurately identified the high-risk population. This study contributes to add novel insights into the biology of cHL and to identify new prognostic features that might be used to define future strategies to improve the management of patients.

Because the concept of early metabolic response was defined (14), iPET has been identified as in vivo chemoresistance assay and used as predictive tool to adapt the intensity of subsequent therapy (3, 4, 6). Indeed, iPET contributed to abrogating most of the individual patient
Figure 4.
Validation of iPET predictive score in an independent cohort of patients with cHL. A, Outline of the workflow for the validation of the iPET predictive score. B, Boxplots representing the expression of genes found associated with iPET+ in the validation cohort. C, ROC curve of the scoring system in the validation cohort of patients with cHL (n = 81, 1 of 82 patient lacks LMR value). D, Boxplot of score distribution between iPET+ and iPET− patients. E, Boxplot of score distribution between TF− and TF+ patients (n = 115).
differs, making it possible to confirm response-adapted therapy as the best treatment modality to optimize the risk-benefit ratio of treatment both in early and in advanced stage. Response-adapted therapy is a reasonable approach to optimize toxicity profile for patients who are iPET after two courses of ABVD, while the identification of high-risk cases at early time points than iPET, represents an unmet clinical need. While being an important decision-making tool, the early assessment of the metabolic response has some limitations. The main limitation is given by the fact that the prognostic information provided by iPET is obtained only after two months of therapy and not at the time of diagnosis. Moreover, while iPET is the strongest prognostic parameter in cHL, it is the result of complex and still unknown interactions between the tumor, the patient, and the treatment whose characterization would likely improve patient management.

Our iPET predictive score is a first answer for the identification of baseline features to predict chemoresistance in cHL. To the best of our knowledge, this is the first study to identify early predictors of chemoresistance as anticipated by iPET. As confirmed by our results, none of the clinical and laboratory parameters was able to predict early response with the only exception of LMR, which was integrated in the final model as an independent covariate. In particular, our model was not influenced by cHL subtype, clinical stage, and patient’s age.

This analysis shows that iPET response in cHLs is influenced by innate biological diversity. iPETþ cHLs are biologically different from iPET– tumors and they rely on the expression of a subset of genes that likely confer aggressiveness and refractoriness to ABVD chemotherapy. Tumor–microenvironment can initiate and support cancer progression (15). cHL is considered a paradigmatic example of the role of microenvironment in cancer (16). Like no other tumors, cHL is characterized by a dominant microenvironmental component and the Reed–Sternberg (RS) cells heavily rely on the paracrine crosstalk with their neighboring cells to survive and progress (17). In line with this evidence, our 13-gene signature is largely representative of stromal interactions. Two distinct but interconnected nodes emerged within this signature. CXCL2, CXCL3, CCL18, TREM1, and SAA1 are well known proinflammatory molecules. CXCL2 and CXCL3 are small chemokines, secreted mainly by monocytes, that exert a chemotactic function for polymorphonuclear leukocytes including neutrophils and macrophages (18, 19). Furthermore, both these molecules are involved in cancer-related mechanisms including wound healing, cancer metastasis, and angiogenesis. As well CCL18 is a CC-chemokine produced by cells of innate immunity like dendritic cells, monocytes, and macrophages and acts as chemoattractant signal for T and dendritic cells (20). CCL18 has been also linked to immune suppression because exposure to CCL18 causes macrophages differentiation the M2 versus iPETþ samples (Supplementary Fig. S4A). This is in line with previous reports that suggest an association between tumor-associated macrophages with iPET response and shortened survival in patients with cHL even if hierarchically less important than iPET (35).

Our work is not the only one trying to use gene expression to anticipate cHL behavior. Recently, Scott and colleagues proposed a 23-gene-based model to predict overall survival in patients with cHL (36). Comparing our results with the one obtained in this work, we observed very little overlap and only 2 of the Scott 23-gene signature (IL15RA and CD68) were significantly associated with iPETþ in our analysis (Supplementary Fig. S4B). Many factors may account for this apparent discrepancy. First, we used a commercial panel comprising 770 immune-related genes that included only 11 of the 23 gene signatures identified by Scott, thus reducing the possibility of comparison among these datasets. In addition, even if we cannot exclude that technical and methodological differences in the study design may have influenced, we believe that the two signatures are quite difficult to compare because they were developed to respond to different questions. We also acknowledge the potential limitations of our model, which mainly concern its reproducibility.

The limited number of patients from two single centers and the overall low number of iPETþ included in this study are relevant limitations to the potential generalization of the results, and warrant further validation. Counterbalancing the small study size, patients’ recruitment from the two centers allowed to grant consecutiveness of enrollment and contributed to a high-quality dataset; indeed, patients were all treated using the same regimens and in the same institution, FDG-PET were done only in two scanners, were read by dedicated nuclear physicians prior to blinded review.

For iPET, we adopted the standard Deauville 5-point score, which is associated with high, although not absolute reproducibility rates (13) and which is now widely used also in daily practice. Even if DS is the recommended tool for response assessment in cHL, some concerns about its accuracy in identifying nonresponding patients have recently been raised; in particular, while confirming the bad prognostic value of DS5, the clinical meaning of DS4 has been questioned (37). The relative inaccuracy of DS4 might have impacted the results of our study, being a potential limitation. However, the observed 100% sensitivity of our iPET predictive score to identify DS5 strengthens our hypothesis and supports the validity of results. Further validation on larger and
prospective series of patients will lead to a better assessment of the model. Additional radiomic parameters that have been recently identified to predict survival in Hodgkin lymphoma, including total metabolic tumor volume, were not evaluated in this study but will be included in larger future analyses. Moreover, our study only applies to patients treated with ABVD regimen. BEACOPP chemotherapy is alternative intensified regimen to ABVD for the initial therapy of patients with advanced cHL. Interestingly, response adapted approach has recently been validated in the setting of this intensive regimen as well but with an inverse approach to what is done with ABVD (i.e., adapting treatment intensity by reducing the number of cycles or by deescalating to ABVD in iPET adapted approach has recently been validated in the setting of this approach). BEACOPP chemotherapy, metabolic tumor volume, were not evaluated in this study but will apply to patients treated with ABVD regimen. BEACOPP chemotherapy is alternative intensified regimen to ABVD for the initial therapy of patients with advanced cHL. The iPET predictive model was developed for the use on NanoString platform. This technology allows accurate and reproducible RNA quantification from FFPE samples and is gaining consideration in clinical diagnostics. Once consolidated by further studies, the iPET predictive score could easily be applied to clinical diagnostics to improve the risk-based stratification of patients with cHL.

In conclusion, similar to other malignant lymphomas (10, 38), biological variation as measured by means of gene expression can be linked to therapy response in cHL. We have established a 5-gene predictive score integrated with LMR to predict the risk of not achieving a complete metabolic response to the first 2 ABVD cycles in cHL. This score can be defined at the time of cHL diagnosis and has the potential to be used to define treatment strategy upfront without waiting 2 months from treatment start. Additional studies are warranted to further validate these results in larger patient populations and in the setting of early response to intensified therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
This work was supported by the 5 x 1000/2014 funding of AUSL-IRCCS Reggio Emilia (to S. Luminari) Fondazione GRADE ONLUS, Reggio Emilia (to A. Ruffini). We also wish to thank Dr. Savonarola for inspiring our writing.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 20, 2019, revised September 12, 2019, accepted October 15, 2019, published first October 23, 2019.

References
1. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood 2016;127:2375–90.
2. Merli F, Luminari S, Gobbi PG, Cascavilla N, Mammi C, Ilarucci F, et al. Long-term results of the HD2000 trial comparing ABVD versus BEACOPP versus COPP-EBV-CAD in untreated patients with advanced Hodgkin lymphoma: a study by Fondazione Italiana Linfomi. J Clin Oncol 2016;34:1175–81.
3. Andre MPE, Girinsky T, Fedorico M, Romeno C, Fortpiet C, Gottì M, et al. Early positron emission tomography response-adapted treatment in stage I and II Hodgkin lymphoma: final results of the randomized EORTC/LYSA/FIL H10 Trial. J Clin Oncol 2017;35:1786–94.
4. Raemakers JM, Andre MP, Fedorico M, Girinsky T, Oumedly R, Brusamolino E, et al. Omitting radiotherapy in early positron emission tomography-negative stage I (II) Hodgkin lymphoma is associated with an increased risk of early relapse: clinical results of the preplanned interim analysis of the randomized EORTC/LYSA/FIL H10 trial. J Clin Oncol 2014;32:1188–94.
5. Borchmann P, Haverkamp H, Lohri A, Mey U, Kreissl S, Greil R, et al. Hodgkin lymphoma: ESMO clinical practice guidelines for diagnosis, based interpretation of early interim PET in advanced-stage Hodgkin lymphoma: a retrospective training and validation analysis in three international cohorts. Lancet Oncol 2018;19:549–61.
6. Manzotti G, Torricelli F, Benedetta D, Lococo F, Sancisi V, Rossi G, et al. An epithelial-to-mesenchymal transcriptional switch triggers evolution of pulmonary sarcomatoid carcinoma (PSC) and identifies dasatinib as new therapeutic option. Clin Cancer Res 2019;25:2348–60.
7. Jensen LJ, Kuhn M, Stark M, Chaffron S, Crevee C, Muller J, et al. STRING 8–a global view on proteins and their functional interactions in 630 organisms. Proc Natl Acad Sci USA 2008;105:1783–8.
8. Eichenauer DA, Aleman BMP, Andre M, Fedorico M, Hutchings M, Illidge T, et al. Hodgkin lymphoma. ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2018;29:v19–29.
9. Hoppe RT, Advani RH, Al WZ, Ambinder RF, Anon P, Armand P, et al. NCCN Guidelines Insights: Hodgkin lymphoma, version 1.2018. J Natl Compr Canc Netw 2018;16:245–54.
10. Huet S, Tesson B, Jaie JP, Feldman AL, Magnano L, Thomas E, et al. A gene-expression profiling score for prediction of outcome in patients with follicular lymphoma: a retrospective training and validation analysis in three international cohorts. Lancet Oncol 2018;19:549–61.
11. Steidl C. The ecosystem of classical Hodgkin lymphoma. Blood 2017;130:2360–62.
12. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucetto E, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol 2014;32:3059–68.
13. Gallamini A, Rigacci L, Merli F, Natti L, Bosi A, Capodanno I, et al. The predictive value of positron emission tomography scanning performed after two courses of standard therapy on treatment outcome in advanced stage Hodgkin’s disease. Haematologica 2006;91:475–81.
14. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
15. Steidl C. The ecosystem of classical Hodgkin lymphoma. Blood 2017;130:2360–1.
16. Liu Y, Sattarzadeh A, Diepstra A, Visser L, van den Berg A. The microenvironment in classical Hodgkin lymphoma: an actively shaped and essential tumor component. Semin Cancer Biol 2014;24:15–22.
17. Al-Abwan LA, Chang Y, Mogas A, Halayko AJ, Baglole CJ, Martin JG, et al. Differential roles of CXCL2 and CXCL3 and their receptors in regulating normal and asthmatic airway smooth muscle cell migration. J Immunol 2013;191:2731–41.
19. Wolpe SD, Sherry B, Juers D, Duvatelis G, Yurt RW, Cerami A. Identification and characterization of macrophage inflammatory protein 2. Proc Natl Acad Sci U S A 1989;86:612–6.
20. Adema GJ, Hartgers F, Verstraten R, de Vries E, Marland G, Menon S, et al. A dendritic-cell-derived C-C chemokine that preferentially attracts naïve T cells. Nature 1997;387:713–7.
21. Schraufstatter IU, Zhao M, Khaldoon A, Discipio RG. The chemokine CCL18 causes maturation of cultured monocytes to macrophages in the M2 spectrum. Immunology 2012;135:287–98.
22. Bouchon A, Facchetti F, Weigand MA, Colonna M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. Nature 2001;410:1103–7.
23. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol 2000;164:4991–5.
24. De Santo C, Arcott R, Booth S, Karydis I, Jones M, Asher R, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. Nat Immunol 2010;11:1039–46.
25. Hansen MT, Forst B, Cremers N, Quagliata L, Ambartsumian N, Grum-Schwensen B, et al. A link between inflammation and metastasis: serum amyloid A1 and A3 induce metastasis, and are targets of metastasis-inducing S100A4. Oncogene 2015;34:424–35.
26. Tomlin H, Piccinini AM. A complex interplay between the extracellular matrix and the innate immune response to microbial pathogens. Immunology 2018;155:186–201.
27. Linke F, Harenberg M, Nettet MM, Zunig S, von Bonin F, Arlt A, et al. Microenvironmental interactions between endothelial and lymphoma cells: a role for the canonical WNT pathway in Hodgkin lymphoma. Leukemia 2017;31:361–72.
28. Steidl C, Connors JM, Gascogne RD. Molecular pathogenesis of Hodgkin’s lymphoma: increasing evidence of the importance of the microenvironment. J Clin Oncol 2011;29:1812–26.
29. Marinaccio C, Nico B, Maiorano E, Specchia G, Ribatti D. Insights in Hodgkin lymphoma angiogenesis. Leuk Res 2014;38:857–63.
30. Cohen EN, Gao H, Anfossi S, Mego M, Reddy NG, Debeeb B, et al. Inflammation mediated metastasis: immune induced epithelial-to-mesenchymal transition in inflammatory breast cancer cells. PLoS One 2015;10:e0132710.
31. Mantuano E, Biffi A, Lam MS, Azmoon P, Gilder AS, Gennia SL. LDL receptor-related protein-1 regulates NFκB and microRNA-155 in macrophages to control the inflammatory response. Proc Natl Acad Sci U S A 2016;113:1369–74.
32. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavrey SJ, et al. An IL-23R/IL-22 circuit regulates epithelial serum amyloid a to promote local effector Th17 responses. Cell 2015;163:381–93.
33. Staudt ND, Jo M, Hu J, Bristow JM, Pizzolo D, Gaultier A, et al. Myeloid cell receptor LRP1/CD91 regulates monocyte recruitment and angiogenesis in tumors. Cancer Res 2013;73:3902–12.
34. Steinman L. A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell-mediated tissue damage. Nat Med 2007;13:139–45.
35. Agostinelli C, Gallamini A, Scarcha G, Agati P, Tripodi C, Fuligni F, et al. The combined role of biomarkers and interim PET scan in prediction of treatment outcome in classical Hodgkin’s lymphoma: a retrospective, European, multicentre cohort study. Lancet Haematol 2016;3:e467–e79.
36. Scott DW, Chan FC, Hong F, Hogic S, Tan KL, Meissner B, et al. Gene expression-based model using formalin-fixed paraffin-embedded biopsies predicts overall survival in advanced-stage classical Hodgkin lymphoma. J Clin Oncol 2013;31:692–700.
37. Barrington SF, Phillips EH, Cousonnell N, Hancock B, Pettengell R, Johnson P, et al. Positron emission tomography score has greater prognostic significance than pretreatment risk stratification in early-stage Hodgkin lymphoma in the UK RAPID study. J Clin Oncol 2019;37:1732–41.
38. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. N Engl J Med 2018;378:1396–407.