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ABSTRACT

Purpose

Early response to ABVD, assessed with interim FDG-PET (iPET), is prognostic for classical Hodgkin's 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-. 13 genes were stringently associated with iPET+. This signature comprises two functionally stromal-related nodes. Lymph mono-ratio (LMR) was also associated to iPET+. In the training cohort a 5-gene/LMR integrated score predicted iPET+ (AUC0.88 95%CI,0.80-0.96). The score achieved a 100% sensitivity to identify DS5 cases. Model performance was confirmed in the validation set (AUC0.68 95%,0.52-0.84). Finally, iPET score was higher in patients with event vs 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.

Translational Relevance

Classical Hodgkin Lymphoma (cHL) is a non-linear, 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 data sets 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, represent a good rationale to move to assess the efficacy of this new tool in prospective clinical trials.

BACKGROUND

Classical Hodgkin's 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, vincristine 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. 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 management of B-cell lymphoma patients for a more appropriate risk-based stratification and management(10).

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

MATERIAL 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 (pts) 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: 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; signed informed consent. A validation set was identified from the Hematology Unit of Careggi Hospital of Firenze using the same criteria. Histological sections of all samples were reviewed by three different pathologists (RV, MZ, RS). The project was approved by the local ethics committees.

Gene Expression Profiling (GEP)

Total RNA was extracted by Maxwell® RSC RNA FFPE kit (Promega) starting from 5 slides of 5µm 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 previously described(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 genes counts were normalized on technical controls and housekeeping genes included in the panel.

Mean count of negative controls plus two standard deviations 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. In order to performed 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 standard deviations 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 log2 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's t test) and the false discovery rate (FDR) obtained by the Benjamini-Yekutieli

method were calculated. Finally, genes were ranked on the basis of FC and FDR setting the FC≥2 and FDR<0.1. Bioinformatic analyses on GEP were conducted by R Software v3.4.3 using the following R packages: ggplot2, ggbiplot (function prcomp), corrplot and topGO. Protein-protein interaction networks were evaluated by STRING(12). Gene expression profiles data are available at the Gene Expression Omnibus (GEO) repository (accession number: GSE132348)

Statistical analyses

All clinical variables included in statistical analysis were dichotomized according to standard criteria. The primary study endpoint was iPET defined by an expert nuclear medicine physician, who applied the Deauville-5-point scale (DS) on iPET; DS 4 or 5 defined iPET+(13). The secondary endpoint was treatment failure (TF), which included treatment change after positive iPET (TC), lack of metabolic response at final PET (fPET), progressive disease (PD), whichever came first. Only patients with at least three years of follow-up were included in TF analysis. Survival function for TF was calculated from time of diagnosis to date of TF or last follow up (time to TF- TTF).

Univariate descriptive statistics were used to compare iPET+ and iPET- patients of both training and validation cohort on the basis of clinical variables. A multivariate regression logistic analysis was conducted to identify variables independently associated with iPET+. Variables with *p*-value<0.08 by multivariate analysis were used to construct a scoring system to predict iPET+.

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. Internal resampling of original data by means of bootstrap (R package rms) was used to assess the validity of the model.

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 cHL patients 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 iPET+ from iPET- cHL patients

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 not iPET images available for revision and was therefore excluded (Figure 1A). Differential analysis between iPET+ and iPET- associated GEP identified 241 (33%) significantly deregulated genes (p-value <0.05) (Figure 1B-D). The majority of these genes (n=171, 71%) were upregulated while 70 genes (29%) were repressed in iPET+ compared to iPET- cHLs. Principal components analysis (PCA) clustered iPET+ and iPET- samples in two separate groups (Figure 1C). Gene Ontology (GO) analysis of the 241 genes highlighted enrichment of many cancer-relevant pathways, including immune response, inflammation and cell migration (Supplementary Figure S1).

In order 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 cut-offs. We identified a list of 13-genes which expression was positively correlated with iPET+ (Figure 1D-E, Table 2). Protein-protein interaction analysis identified two stromal-related nodes within the 13-gene signature (Figure 2A). The first comprised microenvironment-related immune-modulatory factors including the chemotactic cytokines CXCL2, CXCL3, and CCL18, the myeloid cells receptor TREM1, and the pro-inflammatory 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 pro-angiogenetic 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 in order to define possible collinearity between genes. We identified ITGA5, CD9, and FN1 as strongly interdependent (correlation coefficient=0.8) (Figure 2B). Due to this collinearity, CD9 and FN1 were excluded and ITGA5 was maintained in further analyses as representative of this node, being the most strongly associated to iPET+ (Table2). Next, multivariate logistic regression 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% CI 0.80-0.96) (Figure 2C-D). Application of the score to the training cohort consistently segregated iPET+ from iPET- cHLs (Figure 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: Q4-76.2%, Q3-9.5%, Q2-14.3% and Q1-0% (Figure 3C). Overall, about 80% of the true iPET+ patients were correctly allocated within Q4, which represents the iPET+ patients as predicted by the score, confirming the discriminatory capacity of the model.

Internal validation with bootstrap resampling was performed (Figure 3D) confirming the elevated performance of the model (AUC 0.84). To further consolidate these data, we explored the distribution of the iPET predictive score according to the DS value (DS1-5) at iPET (Figure 3E). Noticeably, while the score value was homogeneous in patients with a DS1-3 it increased consistently in patients with DS4-5. The average iPET predictive score in DS5 was higher and less dispersed than in DS4, even if this difference was not statistically significant. Finally, 100% of DS5 patients included in the analysis scored positive according to our model and allocated in Q4 (Figure 3F). We also investigated whether the iPET predictive score associated with additional clinical features at diagnosis. No significant correlation with histotype, age, stage and risk group was observed (Supplementary Figure S2A-B). However, a positive correlation between the iPET predictive score and the SUVmax value at diagnosis was observed (Figure 3G). The gene-based iPET score showed a higher performance in predicting iPET+ with respect to SUVmax value at baseline (Figure 3H).

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 (Figure 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-genes stromal signature in iPET+ and iPET- patients and confirms their association with iPET+. By contrast no significant differences were observed in the expression of these genes in the iPET+ groups comparing the two cohorts (Supplementary Figure S2C). ROC analysis obtained an AUC of 0.68 (0.52-0.84), specificity 69% sensibility 64% accuracy 68% (Figure 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) (Figure 4D) confirming the validity of the model.

iPET+ predictive score and treatment failure

We conducted an exploratory analysis with the aim of assessing the potential association of the iPET predictive score with Treatment Failure (TF) within the entire cohort (training and validation). Treatment Failure (TF) was defined as one of the following: change of therapy after iPET+ (TC), lack of metabolic response at final PET (fPET), progressive disease (PD), whichever came first. Only patients with at least three 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), fPET+ (n=12) and PD (n=3). Boxplot distribution demonstrated that the iPET predictive score was significantly higher in TF+ vs TF- cHL patients (p=0.02) (Figure 4E). Finally, we correlated the iPET score with Time to TF (TTF). The median follow-up of our series was 36 months (range 2 to 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 Figure S3A). We also investigated the iPET predictive score in patients that experienced clinical events without considering the treatment change as treatment failure. (Supplementary Figure 3B). In this case no difference was observed. Administered treatments in both training and validation sets are summarized in Supplementary Figure S3C.

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 gene expression profile of a consecutive series of cHL patients 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.

Since the concept of early metabolic response was defined(14), iPET has been identified as in vivo chemorefratoriness 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 differences, 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 earlier 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 chemorefractoriness in cHL. To the best of our knowledge, this is the first study to identify early predictors of chemorefactoriness 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. iPET+ 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 micro-environmental 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 pro-inflammatory 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 since exposure to CCL18 causes macrophages differentiation the #M2 spectrum, which promotes immunosuppression and healing (21). TREM1 is a super-immunoglobulin receptor expressed exclusively on myeloid cells. This protein amplifies neutrophil and monocyte-mediated inflammatory responses largely by stimulating release of pro-inflammatory chemokines and cytokines. Recent evidence links TREM-1 to tumor-associated macrophages implying its relationship to tumor growth and progression (22, 23). SAA1 is a major acute-phase protein that is highly expressed in response to inflammation and tissue injury and it is largely controlled by inflammation-associated cytokines (24, 25).

Immune cell recruitment as well as cancer progression rely on cell ability to migrate and infiltrate surrounding tissue. Indeed, the second node within the 13—gene signature comprises proteins involved in cell movement (like ITGA5, THBS1, LRP1 and PLAU) and matrix organization (FN1, SPP1). Many of these genes have been already described as marker of cancer aggressiveness even in the setting of B-cell Lymphoma. FN1, SPP1 and LRP1 have been reported to partake to immunomodulatory responses, in particular by recruiting cells to inflammatory sites (26). Noticeably, VEGFA, master regulator of angiogenesis, seems to bridge the connection between the two functional nodes identified within the iPET+ predictive signature. Increased VEGFA levels in cHL secretome mediate endothelial cells recruitment to the tumor (27). Furthermore, high

VEGFA expression has been shown to correlate with reduced overall survival in cHL patients (27, 28), underscoring the relevance of angiogenesis-related factors in priming cHL aggressiveness(29). Indeed, many of the 13-genes of the signature (including CXCL2, CXLC3 FN1, THSB1, PLAU and LRP1) have been shown to partake to angiogenesis regulation also in the context of cancer. Thus, together, the augmented expression of these genes may underscore increased aggressiveness in cHLs (18, 19, 22, 23, 25, 30-34). As well, the expression of the 13-gene iPET+ signature is likely to reflect increased macrophages recruitment and activity in iPET+ cHLs, which is further confirmed by the fact that in our dataset, tumor associate macrophage associated markers were found to be consistently up-regulated in iPET+ vs iPET- samples (Supplementary Figure 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 is not the only work trying to use gene expression to anticipate cHL behavior. Recently, Scott and colleagues proposed a 23 gene-based model to predict overall survival in cHL patients (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 iPET+ in our analysis (Supplementary Figure 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 signature identified by Scott, thus reducing the possibility of comparison among these data sets. 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 since they were developed to respond to different endpoints.

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 iPET+ 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 data set; 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, though 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 non-responding patients have recently been raised; in particular, while confirming the bad prognostic value of DS5 the clinical meaning of DS 4 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 HL, 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 de-escalating to ABVD in iPET-patients). A validation of our genetic signature in patients initially treated with BEACOPP is warranted.

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 cHL patients.

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.

AUTHOR CONTRIBUTIONS

BD and VF performed experiments, BD performed data analysis, CM, RV, RS, BP, KS, AF,VB, MZ, FV and AV collected clinical data, AR managed data collection and organization, LR and FM supervised clinical analysis, SL and AC designed study, SL, BD and AC analyzed data, SL, BD and AC wrote the manuscript.

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

		TRAINING COHORT				VALIDATION COHORT			
		iPET response				iPET response			
Variable	**Status**	**Pos (n)**	**Neg (n)**	***p***	**adj *p***	**Pos (n)**	**Neg (n)**	***p***	**adj *p***
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%)			8 (34.8%)	29 (33.0%)		
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%)			8 (34.8%)	46 (52.3%)		
Leukocytes (x 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%)			6 (26.1%)	9 (10.2%)		
Lymphocytes (x 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%)			21 (91.3%)	85 (96.6%)		
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%)			11 (47.8%)	45 (51.1%)		
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%)			17 (73.9%)	83 (94.3%)		
LDH/ULN	**<1**	15 (65.2%)	63 (64.9%)	0.80	0.14	12 (52.2%)	55 (62.5%)	0.58	0.61
	>1	6 (26.1%)	31 (31.9%)			7 (30.4%)	22 (25.0%)		
ESR (mm/h)	**<50**	5 (21.7%)	46 (47.4%)	**0.03**	0.21	13 (56.5%)	50 (56.8%)	0.61	0.47
	>50	17 (73.9%)	46 (47.4%)			8 (34.8%)	24 (27.3%)		
STAGE	**I-II**	8 (34.8%)	54 (55.7%)	0.11	0.99	9 (39.1%)	52 (59.1%)	0.14	0.37
	III-IV	15 (65.2%)	43 (44.3%)			14 (60.9%)	36 (40.9%)		
Symptoms	**A**	14 (60.9%)	61 (62.9%)	>0.99	0.67	12 (52.2%)	59 (67.0%)	0.28	0.97
	B	9 (39.1%)	36 (37.1%)			11 (47.8%)	29 (33.0%)		
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%)			10 (43.5%)	14 (15.9%)		
Risk Group *	**early**	8 (34.8%)	50 (51.5%)	0.22	0.99	1 (4.3%)	15 (17.0%)	0.57	-
	advanced	15 (65.2%)	47 (48.5%)			2 (8.7%)	11 (12.5%)		

		TRAINING COHORT				VALIDATION COHORT			
		iPET response				iPET response			
Variable	**Status**	**Pos (n)**	**Neg (n)**	***p***	**adj *p***	**Pos (n)**	**Neg (n)**	***p***	**adj *p***

Age	**<45**	15(65.2%)	58 (59.8%)	0.81	0.12	15 (65.2%)	56 (66.7%)	>0.99	0.16
	>45	8 (34.8%)	39 (40.2%)			8 (34.8%)	28 (33.3%)		
Sex	**Male**	11 (47.8%)	49 (50.5%)	>0.99	0.95	15 (65.2%)	40 (47.6%)	0.21	0.93
	Female	12 (52.2%)	48 (49.5%)			8 (34.8%)	44 (52.4%)		
Leukocytes (x 10^3 cells/mm^3)	**<15**	17 (73.9%)	87 (89.7%)	**0.03**	0.25	17 (73.9%)	74 (88.1%)	0.09	0.35
	>15	6 (26.1%)	8 (8.2%)			6 (26.1%)	9 (10.7%)		
Lymphocytes (x 10^3 cells/mm^3)	**<0.6**	2 (8.7%)	10 (10.3%)	>0.99	0.97	2 (8.7%)	1 (1.2%)	0.12	0.70
	>0.6	21 (91.3%)	85 (87.6%)			21 (91.3%)	81 (96.4%)		
LMR	**<2.1**	17 (73.9%)	42 (43.3%)	**0.02**	**0.05**	12 (52.2%)	40 (47.6%)	0.82	0.36
	>2.1	6 (26.1%)	53 (54.6%)			11 (47.8%)	42 (50.0%)		
Hemoglobin g/dl	**<10.5**	2 (8.7%)	16 (16.5%)	0.52	0.15	6 (26.1%)	3 (3.6%)	**0.003**	**0.05**
	>10.5	21 (91.3%)	79 (81.4%)			17 (73.9%)	80 (95.2%)		
LDH/ULN	**<1**	15 (65.2%)	63 (64.9%)	0.80	0.14	12 (52.2%)	55 (65.5%)	0.38	0.93
	>1	6 (26.1%)	31 (31.9%)			7 (30.4%)	18 (21.4%)		
ESR (mm/h)	**<50**	5 (21.7%)	46 (47.4%)	**0.03**	0.21	13 (56.5%)	49 (58.3%)	0.59	0.51
	>50	17 (73.9%)	46 (47.4%)			8 (34.8%)	21 (25.0%)		
STAGE	**I-II**	8 (34.8%)	54 (55.7%)	0.11	0.99	9 (39.1%)	51 (60.7%)	0.11	0.29
	III-IV	15 (65.2%)	43 (44.3%)			14 (60.9%)	33 (39.3%)		
Symptoms	**A**	14 (60.9%)	61 (62.9%)	>0.99	0.67	12 (52.2%)	58 (69.0%)	0.21	0.94
	B	9 (39.1%)	36 (37.1%)			11 (47.8%)	21 (25.0%)		
Bulky	**no**	19 (82.6%)	82 (84.5%)	0.76	0.54	13 (56.5%)	69 (82.1%)	**0.01**	0.09
	yes	4 (17.4%)	15 (15.5%)			10 (43.5%)	14 (16.7%)		
Risk Group *	**early**	8 (34.8%)	50 (51.5%)	0.22	0.99	1 (4.3%)	14 (16.7%)	0.54	-
	advanced	15 (65.2%)	47 (48.5%)			2 (8.7%)	8 (9.5%)		

Abbreviations: LMR (lymphocytes/ monocytes ratio), PD (disease progression), LDH/ULN (Lactate dehydrogenase/upper limit normal), ESR (erythrocytes sedimentation rate), adj pVal (adjusted for all clinical variables considered in the table), * not included in multivariate analysis for validation cohort since value was missing for 88 patients (78%).

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*-value	FDR	Multivariate *p*-value	Multivariate *p*-value including LMR
VEGFA	NM_001025366.1	2.02	1.566E-07	0.0008	0.504	0.649
PLAU	NM_002658.2	2.07	4.850E-06	0.008	0.972	0.500
THBS1	NM_003246.2	2.35	1.004E-04	0.055	0.899	0.952
ITGA5	NM_002205.2	2.67	1.294E-04	0.062	0.034 ◊	0.023 ◊
SAA1	NM_199161.1	5.80	1.701E-04	0.063	0.139	0.072 ◊
FN1*	NM_212482.1	3.92	1.553E-04	0.063	-	-
LRP1	NM_002332.2	2.22	2.053E-04	0.063	0.202	0.100
CXCL2	NM_002089.3	2.90	2.347E-04	0.065	0.018 ◊	0.008 ◊
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	0.094 ◊	0.060 ◊
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	0.079 ◊
LMR						0.055 ◊

Abbreviations: FC (fold change), FDR (false discovery rate) calculated by Benjamini-Yekutieli method, LMR (Lymphocytes/ monocytes ratio), * genes excluded from multivariate analyses on the basis of correlation matrix result (Fig.2B), ◊ genes included in the final model.

FIGURE LEGENDS

Figure 1. Lesions of iPET-positive 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- lesions. Black dots represent genes significantly deregulated (p-value ≤ 0.05) and dashed lines indicate absolute FC≥2. C) Principal component analysis (PCA) shows the variance between iPET+ (black dots) and iPET- (grey dots) samples explained by the 241 genes differentially expressed. D) Summary of genes differentially expressed between iPET+ and iPET- patients, using progressive selection criteria. E) Boxplots representing the expression of the 13 genes associated with iPET+ in the training cohort.

Figure 2. Building of a gene-based iPET predictive score

A) String and Gene Ontology (GO) combined analyses built on the iPET+ 13-genes signature. String analysis reveals two principal stromal-related clusters associated to matrix interaction and deposition (red) and immune response and inflammation (green) by GO analysis. B) Correlation within the iPET+ 13-genes signature. Colors and size of the dots are proportional to correlation coefficients, thus representative of the strength of the dependence. C) ROC Curve of the scoring system in the training cohort of cHL patients (n=104, 1/106 patient lacks iPET result and 1/106 lacks LMR value). Curve is colored according to score values, relation between colors and different score values is represented by the bar on the right. Formula has been generated by generalized linear regression model. D) Table shows sensitivity, specificity, accuracy, positive predictive values (PPV) and negative predictive value (NPV) for different score threshold. Best threshold was identified at -0.93 (highlighted in blue).

Figure 3. iPET predictive score segregates iPET+ from iPET- cHL samples. A) Boxplot of score distribution between iPET+ and iPET- patients. B) Contribution of each gene to the score is indicated by the scatter plot where each dot represents the mean expression of the gene multiplied by the coefficient assigned to the gene in the score. The heatmap shows the relative expression (iPET+ vs iPET-) of each gene (labels are reported in the plot above). Each row (n=104) of the heatmap represents a patient ranked by predictive score value. LMR and iPET data are also reported; and each line represents a patient with LMR >2.1 (green) or iPET positive (red). C) Patients distribution into score quartiles according to iPET results. D) Bootstrap calibration plot showing actual versus predicted probability of iPET positivity (AUC 0.84, B=1000, Mean absolute error = 0.044, n=104). E) Box-plot of score distribution within each DS category (DS1-5). DS1-3 are iPET- while DS4-5 are iPET+. F) Distribution of model-predicted iPET+ patients (Q4) according to DS. 100% of DS5 were correctly predicted by the model, while 10 out of 87 iPET-(DS1-3) (overall 11%) of DS1-3 were not correctly assigned. G) Sperman correlation between SUV max at diagnosis and iPET predictive score. H) ROC Curves of the iPET predictive score (red) and SUV Max value (black) in predicting iPET+ (SUVmax AUC=0.69; CI 95% 0.56-0.82).

Figure 4. Validation of iPET predictive score in an independent cohort of cHL patients. 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 cHL patients (n=81, 1 out 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).