Introduction

The great majority of patients with Hodgkin lymphoma (HL) can be cured with chemotherapy or a combination of chemo and radiotherapy. There is, however, a proportion of patients, in particular those presenting with advanced stage disease, who will succumb to the disease [1]. Balancing the aggressiveness of treatment between disease control and risk of short- and long-term toxicity remains a challenge for treatment decisions in HL [2]. Aggressive treatment of advanced stage disease using the BEACOPP regimen has certainly improved disease-free survival, at the cost of infertility and risk of secondary organ damage and neoplasias [3–7]. Classical clinical and laboratory risk factors at diagnosis appear to be of little help for treatment decisions in patients with advanced HL [8, 9].
In 2006, Gallamini et al. and Hutchings et al. reported that PET examination with $^{[18}F]$fluorodeoxyglucose (FDG) after 2 cycles of standard chemotherapy, later on termed “interim positron emission tomography (PET)”, discriminates PET-negative patients with a very high probability of disease control with the standard chemotherapy regimen Adriamycin, Bleomycin, Vinblastine, Dacarbazine (ABVD), from PET-positive patients where standard therapy is most likely to fail [10, 11]. These data were confirmed by several studies on patient groups with limited or advanced stage disease treated with ABVD, while the prognostic value of interim PET for patients treated with BEACOPP is not well established [12–15]. During a consensus meeting at Deauville in France, criteria have been standardized to evaluate interim PET, by using a 5-point scale [16]. The 5-point scale uses uptakes by the mediastinal blood flow and the liver to quantify residual uptake in a visual evaluation. The Deauville criteria are now widely considered as the most appropriate evaluation method for interim PET [17]. A PET-guided treatment approach allows the early identification of interim PET-positive patients, with insufficient response to standard treatment, as candidates for intensive, although potentially more toxic, treatments [18]. This approach is currently evaluated in prospective studies. A drawback of the PET-guided approach is that patients with poor prognostic features are only identified after 2 months of treatment, significantly delaying intensive treatment choices. In addition, there is a small, but consistent proportion of interim PET-negative patients who will progress or relapse, with a progression-free survival (PFS) around 80–85% as indicated by recent preliminary data [19]. This leaves room for other potential prognosticators in addition to interim PET. For patients with refractory/relapsed HL, Moskowitz et al. showed that involvement of extranodal sites and a positive PET result pre-high-dose therapy were independent risk factors [20].

A particular feature of HL is that the neoplastic cells vitally depend on the supporting microenvironment. The cellular composition of the microenvironment impacts prognosis in HL. In 2010, a gene expression study by Steidl et al. pointed to the prominent role of tumor-infiltrating macrophages in HL lymphnode biopsies [21]. Over 5% tumor-infiltrating macrophages identified by immunohistochemical staining for the CD68 antigen pick out patients at higher risk for PFS. The number of CD68+ macrophages outperformed the international prognostic score (IPS) in multivariate analysis. These data have been confirmed by several groups, including ours [22–29]. CD68+ cell counts appear as the most reproducible and simple prognostic marker reflecting tumor biology and is currently available, using routine diagnostic methods.

Another common feature of the tumor microenvironment in HL is the overrepresentation of tolerogenic T-cell populations, that include T helper 2 (TH2) cells and regulatory T cells (Treg). These cells create a favorable immunological environment for the survival and proliferation of HRS cells. The chemokine thymus and activation-regulated chemokine (TARC), also termed CCL17, engages the chemokine receptor CCR4 expressed on regulatory T and TH2 cells, recruiting these cells into HL lesions. TARC is highly expressed by HRS cells, secreted into the serum, and can be detected at high levels at HL diagnosis [30–32]. Recent data suggest that early changes in TARC levels during chemotherapy may be a biomarker for response evaluation [33, 34].

We studied whether microenvironment CD68+ cell counts and TARC levels at HL diagnosis and following 2 cycles of ABVD add prognostic information to interim PET.

**Materials and Methods**

**Patient characteristics**

Our analysis included 102 patients (median age 38 years, range 15–74 years; 47 females and 55 males), diagnosed with classical HL and treated between February 2007 and January 2014 at the Department of Hematology of the Catholic University in Rome. Patient characteristics, including the IPS score [35] are detailed in Table 1. All patients received chemotherapy according to the ABVD protocol. Patients with limited stage disease received 3 or 4 ABVD cycles, according to the presence of other risk factors as defined by the EORTC [36], followed by involved-field radiotherapy. Patients with advanced stage disease received 6 cycles of ABVD. Only 3 patients with advanced stage disease, with a positive interim PET result (score 4–5 according to Deauville criteria) after 2 cycles of ABVD, were switched to BEACOPP (6 cycles of dose-escalated BEACOPP). Two of them did not achieve metabolic remission after the second-line treatment. Radiotherapy was included for consolidation in patients with a limited-stage disease and initial bulky disease. Informed consent was obtained from patients according to institutional guidelines. The study has been approved by the Institutional review board.

**Immunohistochemical analysis**

Immunohistochemical analysis for CD68 was performed on 3 μm tissue slides, using the antihuman mouse monoclonal antibody CD68 (1:100, clone PGM-1, Dako, High Glostrup, Denmark) after proteolytic treatment (pronase 0.05% in tris buffer pH 7.6) for 10 min at room temperature. Immunodetection was performed using an avidin–biotin–peroxidase complex solution (ScyTek, Logan,
UT), 3,39-diaminobenzidine as the chromogen, and Mayer hematoxylin as the counterstain. We used the immunohistochemical score proposed by Steidl et al. with a cut-off at 5% CD68-positive cells [21].

### ELISA for plasma TARC levels

Plasma samples were collected prior to treatment start and at interim PET, and stored at −70°C. TARC levels were determined using a sandwich enzyme-linked immunoassay, according to the manufacturer’s instructions (Human CCL-17/TARC DuoSet, R&D Systems, Inc., Minneapolis, MN). A group of 63 healthy individuals (29 males, 34 females; median age 33 years) was used as control group.

### Interim PET

PET-CT studies were performed using an integrated PET-CT device (GEMINI GXL distributed by Philips Medical System or BIOGRAPH distributed by Siemens. PET-CT images were evaluated by two independent nuclear medicine physicians, using a dedicated fusion and display software (SYNTEGRA by Philips, Milan, Italy or SYNGO. VIA by Siemens, Milan, Italy).

Interim PET was performed after the second ABVD course, few days before the third course. The criteria for PET-2 interpretation were based on visual assessment of FDG uptake, and scored for intensity of FDG uptake according to the Deauville 5-point scoring system [16, 17]. Interim PET scans with a score of 4 that equals a FDG uptake that moderately exceeds the FDG uptake in the liver, and 5 (markedly increased uptake > liver and/or new lesions related to lymphoma) were considered positive.

### Statistical analysis

Fisher’s exact test was used to examine for differences in patient characteristics according to interim PET and the CD68+ cell count. Wilcoxon signed rank test was used for two-sample comparisons of TARC plasma levels, as between patient and control groups, or according to dichotomized patient characteristics. The primary survival end point was PFS, with progression during treatment, lack of complete remission at the end of first-line treatment, relapse, and death from any cause counted as adverse events. A positive interim PET result in the absence of progression that lead to change in therapy from ABVD to BEACOPP in 3 patients was not counted as event. Survival curves were estimated using the Kaplan–Meier product limit method. Log-rank tests were used to analyze for differences in PFS. Hazard ratios and 95% confidence intervals were adjusted for multiple prognostic factors using the Cox proportional hazards model. All parameters that resulted significant (P < 0.05) in the univariate analysis were included into the multivariate analysis. These factors were: interim PET result, stage of disease, presence of B-symptoms, IPS score, and CD68 count. In order to optimize the prognostic model, we performed a stepwise model selection using the Akaike information criterion (AIC). Computations were performed using the Stata 10.0 software (Stata Corp., College Station, TX).

### Results

#### Interim PET

Interim PET-CT scans following 2 ABVD cycles were scored according to the Deauville scoring system in 102 patients with classical HL. In 2 patients, significant FDG accumulation within brown fat tissue did not allow to discriminate for metabolic activity in sites of previous disease. The interim PET scan was scored negative...
(Deauville score 0–3), in 85 patients (83%), while it was positive in 15 patients (15%). Looking at patients’ characteristics, there was a significant association between interim PET-positivity, advanced stage of disease ($P = 0.002$), presence of B-symptoms at diagnosis ($P = 0.02$) and an IPS score >2 ($P = 0.001$) (Table 1).

**TARC levels at diagnosis and interim PET**

TARC levels were determined at diagnosis in 80 patients, and were significantly higher than those of controls ($P > 0.001$) (Fig. 1). TARC levels were above the normal range (upper limit 162 U/mL) at diagnosis in 89% (71/80) of patients with cHL. Significantly higher TARC levels were observed in patients younger than 45 years ($P = 0.02$), and patients with bulky disease ($P = 0.02$) (Table 2). Plasma was available at the time of interim PET in 65 patients. TARC levels decreased at interim PET in 57 patients, but persisted elevated (>162 U/mL) in 12 patients (18%) (Fig. 2). Persistently elevated TARC levels were significantly associated with a positive PET result ($P = 0.007$) (Fig. 2).

**Association of the number of CD68+ macrophages with patient characteristics and interim PET results**

Tumor biopsies of 79 patients at the time of HL diagnosis were stained for the CD68 antigen and scored using the immunohistochemical system proposed by Steidl et al. [21]. Over 5% CD68 positive cells were counted in 39/79 (49%) patients. CD68+ cell counts >5% were frequent in patients aged over 45 years (17/22, 77%), when compared to younger patients (24/59, 41%) ($P = 0.005$). There were no other associations between CD68+ cell count and patient characteristics as listed in Table 1.

We observed a direct correlation between interim PET score and CD68 cell counts in the tumor biopsy. When grouping patients according to the consensus cut for PET-positivity, which is score 4 [16], the association between a positive interim PET and higher CD68 counts was not significant (8/39, 21% vs. 4/40, 10%; $P = 0.2$). On the other hand, patients with interim PET score 1 or 2 according to the Deauville system frequently had less than 5% CD68+ counts, while patients scored 3–5 had significantly higher CD68+ cell counts at diagnosis ($P < 0.05$) (Table 3).

**Associations of CD68+ cell count and interim PET with outcome**

At a median follow-up of 32 months (range 4–88 months), 23 of 102 patients had disease progression, which translated into a 77% probability of PFS (95% CI, 67–84%). The univariate analysis showed that positive interim PET was associated with significantly worse PFS ($P < 0.0001$) (Fig. 3A), as was the number of CD68+ macrophages ($P = 0.006$) (Fig. 3B), advanced stage of disease ($P = 0.008$), presence of B-symptoms ($P = 0.0002$, Fig. 3C) and IPS

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**Table 2.** Thymus and activation-regulated chemokine (TARC) levels according to patient characteristics.

| Parameter       | Variable | Cases ($n = 80$) | TARC (U/mL) median | $P^{1}$  |
|-----------------|----------|------------------|--------------------|----------|
| Age             | <45 years| 58               | 2613               | 0.02     |
| Gender          | Female   | 40               | 2571               | 0.07     |
|                 | Male     | 40               | 949                |          |
| Histologic subtype | NS     | 70               | 1885               | 0.1      |
|                 | Others   | 10               | 661                |          |
| Stage           | Limited  | 42               | 1100               | 0.08     |
|                 | Advanced | 38               | 2612               |          |
| B-symptoms      | No       | 55               | 1182               | 0.2      |
|                 | Yes      | 25               | 2059               |          |
| Bulk >5 cm      | No       | 31               | 942                | 0.02     |
|                 | Yes      | 47               | 3829               |          |
| IPS             | IPS 0–2  | 61               | 1482               | 0.7      |
|                 | IPS 3–7  | 19               | 2554               |          |
| CD68+           | <5%      | 30               | 3206               | 0.1      |
|                 | >5%      | 33               | 924                |          |

$^{1}$P-value of Wilcoxon ranked sum test that was used for comparison of TARC levels according to patient characteristics. Significant P-values are shown in bold.
Figure 2. Elevated Plasma levels of thymus and activation-regulated chemokine (TARC) at diagnosis predicted interim positron emission tomography (PET) results. TARC levels were elevated at interim PET in only 6 of 51 (12%) patients with a negative interim PET (A), while they persisted elevated in 6 of 12 (50%) patients with a positive interim PET (B, $P = 0.007$). The dashed line indicates the upper normal value.

### Table 3. CD68+ cell count and interim positron emission tomography (PET).

| Interim PET (Deauville score) | CD68+ <5% | CD68+ >5% | Total no. of patients |
|------------------------------|----------|----------|----------------------|
| 1                            | 18       | 15       | 33                   |
| 2                            | 17       | 9        | 26                   |
| 3                            | 1        | 7        | 8                    |
| 4                            | 4        | 7        | 11                   |
| 5                            | 0        | 1        | 1                    |
| Total                        | 40       | 39       | 79                   |

*P* < 0.05, Fisher’s exact test. Patients with CD68 counts <5% had lower Deauville scores at interim PET when compared to patients with CD68 counts >5%.

### Discussion

We report that the number of CD68+ tumor-infiltrating macrophages and presence of B-symptoms are prognostic markers in ABVD-treated HL also in the era of interim PET, while changes in TARC levels do not add prognostic information. More importantly, this is the first study showing that the integration of interim [18F]-FDG-PET/CT scan results after 2 chemotherapy cycles with presence of B-symptoms and CD68+ cell counts at diagnosis, improves risk-stratification of patients with HL. This additional information may help to identify a group of low-risk patients for whom interim PET is not predictive, and a high-risk patients group who are still at risk also when PET is negative. In our study, the interim PET evaluation was performed according to the recommended 5-point Deauville scale [17]. PET results in our patients are in line with other recent reports on 84% PFS of interim PET-negative patients [19]. However, these figures are lower than initial studies, who reported more than 90% PFS in interim PET-negative patients [10, 11, 19].

We found that TARC plasma levels were elevated at diagnosis in the vast majority of patients with classical HL, and turned to normal following 2 cycles of ABVD. Persistently elevated TARC levels were associated with a higher risk of a positive interim PET. This is in line with two recent
studies suggesting that early changes of TARC may be a useful blood biomarker [33, 34]. However, in our study absolute TARC levels or their changes did not add prognostic information to interim PET. Studied with larger patient numbers and events are required to address this issue.

Macrophage count was higher than 5% in about half of the patients, and was associated to patients’ age over 45 years. This is in line with our previous observation, indicating that the number of CD68+ cells is higher in EBV-associated HL that is typically more frequent in older
patients [22]. Patients with >5% CD68+ cell counts were likely to have a higher (≥3) Deauville score at interim PET. A Deauville score of 3 defines a residual FDG uptake higher than the mediastinal blood flow, but not exceeding activity in the liver. Potential associations between the CD68 count in the microenvironment and PET results in HL have been recently addressed, with conflicting results. Touati et al. [28], reported that the frequency of CD68+ cells correlates to interim-PET results. In contrast, Agur et al. [29] reported that CD68 counts correlate to the initial tumor mass and residual tumor size, but not to interim PET result and PFS. We think that homogenous treatment, standardized scoring of interim PET according to Deauville criteria, and CD68 evaluation by an expert hematopathologist, eliminating inter-observer variability are key issues of quality in our study.

The direct correlation between number of CD68+ macrophages and Deauville score at interim PET may indicate that lymphomas with higher initial macrophage content are at higher probability of treatment resistance, or that persisting macrophages contribute to residual accumulation of [18F]-FDG. On the other hand, one could also speculate that persisting macrophages stimulate metabolic activity of HRS cells.

We found that the presence of B-symptoms is an independent prognosticator in the multivariate analysis. B-symptoms are associated with a variety of other laboratory abnormalities and patient characteristics, in particular advanced-stage disease, and therefore have been often removed in multivariate analyses models, as in the IPS [35]. B-symptoms are due to the production of pro-inflammatory cytokines by the Hodgkin tumor tissue, in particular IL-1, TNF-alpha, and IL-6, which are detected at increased levels in peripheral blood [37]. We found that TARC levels are not predictive of outcome. On the other hand, cytokine models including IL-6, sCD30 and TNFR1 levels was more predictive than the standard clinical score [37]. It will be of great interest to explore whether in the multivariate analysis including CD68+ cell counts and interim PET, the cytokine score could beat clinical characteristics as B-symptoms.

Our study shows that histological (CD68 counts) and clinical characteristics at diagnosis provide prognostic information already at treatment start, and not only after 2 cycles of ABVD. Patients with low CD68+ cell counts and without B-symptoms may have a favorable outcome, despite a positive interim PET scan. On the other hand, patients with high CD68+ cell counts and B-symptoms are at high risk, and treatment results may be poor also in the presence of a negative interim PET. It remains to be determined whether this group could benefit from

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**Table 4.** Multivariate Cox analysis of progression-free survival of 79 patients with HL, treated with ABVD.

| Variable          | Hazard ratio | 95% CI   | P*  |
|-------------------|--------------|----------|-----|
| PET Positive versus negative | 7.5          | 2.3–24.7 | 0.001 |
| CD68 >5% versus <5% | 4.3          | 1.4–13.5 | 0.01 |
| Stage III-IV versus I-IIA | 0.8          | 0.2–3.3  | 0.8  |
| B-symptoms Yes versus no | 4.4          | 1.1–18.6 | 0.04 |
| IPS score >2 versus 0–2 | 0.8          | 0.3–2.5  | 0.8  |

Significant P-values are shown in bold.

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**Figure 4.** Progression-free survival according to interim positron emission tomography (PET) scan in patients according to CD68+ cell count and B-symptoms. PFS curves are shown for patients with CD68 <5% and no B-Symptoms (A), CD68 > 5% or B-symptoms (B), and CD68% >5 and B-symptoms (C). The continuous line indicates patients with negative interim PET, the dashed line patients with a positive interim PET. The survival difference is significant in patients with both CD68 counts >5% and B-symptoms (P = 0.003), while there is a trend for significance in the group with either CD68 counts>5% or B-symptoms (P = 0.06).

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intensified treatment strategies, as the BEACOPP regimen, or addition of new agents, as brentuximab vedotin, right from therapy start. In advanced HL, we are still in need of markers that are not only prognostic, but predictive and can help to tailor first-line therapeutic approaches.

In conclusion, the frequency of CD68+ macrophages at HL diagnosis remains a significant prognostic marker. Patients with a CD68 count >5% and B-symptoms are at high-risk for early progression. It is important to underline that the numbers of this subgroup analyses are small and have therefore to be considered explorative. Technological improvements in the near future using the nanostring technology will probably render the evaluation of the tumor-associated macrophage count more robust and overcome some variability due to the use of different antibody clones (KP1 and PGM1), varying thresholds and inter-observer differences [38]. Moreover, validation of our data on a potential prognostic algorithm integrating information at diagnosis and response evaluation according to interim PET-CT may provide the basis for innovative risk-adapted treatment protocols in HL.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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