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Research Article

Evaluation of Response to Treatment in Mycosis Fungoides. Flow Cytometry – Effectiveness in Everyday Practice

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Abstract

Background: Mycosis fungoides (MF) is difficult not only to diagnose, but also to correctly assess the response. In clinical practice, a physical examination is still important, as well as the subjective feeling of the quality of live. For primary disease staging, flow cytometry (FC) and an assessment of the lymph nodes are recommended. Observation and monitoring of the 3 areas of the skin, lymph nodes and blood, should be carried out during therapy. There is a sense of frustration among dermatologists and oncologist regarding the challenges in interpreting flow cytometry (FC) reports. The most specific and sensitive method is not determined. There are very little data concerning this topic. **Aim:** Analysis of the clinical utility of cytometry for response evaluation. **Material and methods:** Data from a large regional cancer centre located in Bydgoszcz in northwest Poland. In our database 123 patients with Mycosis fungoides diagnosis were identified. The MF database has been maintained since 2007 till now. 396 FC were carried out. The degree of peripheral blood involvement by MF/SS cells was staged as B0, B1 and B2 according to Olsen et al., (2007) and Swerdlow et al., (2017). For evaluation of the blood response criteria, the EORTC recommendations published in 2018 (EORTC 2018) were used, and these results were compared with the EORTC recommendations published in 2011 (EORTC 2011). Patients who have started a new line of therapy after progression in 2018-2019 were chosen for the analysis of the response to cytometry and PET/CT scan carried out simultaneously. The response evaluation of the PET/CT was carried out according to the criteria published by Wahl 2009 and Cheson 2014. **Results:** We present the primary staging, including the flow cytometry (FC) of 104/123 patients, 68/123 patients who were eligible to be evaluated for the fluctuation of atypical cells during observation and 45/123 to be evaluated for the response rate in PET/CT in correlation with cytometry. The complete remission (according to EORTC 2018, T cell blood clone CD4+CD7- or CD4+CD26- were not observed in our database independently of the type of therapy, stage of disease even in nodal/skin remission. Partial regression was observed only in 10.29% of treated patients. PET/CT response evaluation, mainly in lymph nodes correlate with the type of response in the skin. **Conclusion:** The majority of patients have stage B0 or B1, in which changes of the level of cell blood clone CD4+CD7- or CD4+CD26- were not important according to EORTC 2018. Subsequent controls did not show the correlation between the level of absolute cell counts and the level of regression or progression. In our material no correlation with the type of therapy were observed. PET/CT scan results correlate with skin evaluations and confirm or do not confirm the response to therapy, in lymph nodes mainly. Fluctuations between B0, B1 reported in FC are clinically not important, they have no prognostic value.

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Keywords: Mycosis fungoides; Flow cytometry; Response evaluation.

Introduction

Monitoring of MF patients' needs regular evaluations of skin, lymph nodes and clonal cells in blood [1,2,3]. The skin condition differs sometimes, changing from one visit to the next, not only because of the primary disease but frequently as a reaction to the various external and internal conditions. Additionally MF is extreme heterogeneous in skin manifestations. Because of this, the objective methods of response measurement are very important. The SWAT and mSWAT scale is used for the skin response [4,5]. The nodal/visceral response can be evaluated by CT scans or better a PET/CT scan [4,5]. The prognostic value of both these assessments is well known as well as the evaluation efficacy of the response [6]. Flow cytometry was incorporated to the TNM classification in 2007 [1,3,4,5]. FC is recommended to specify the stage and prognosis of mycosis fungoides and Sezary Syndrome, and is also recommended for response evaluation [2,3,5,6,7]. Disease monitoring by blood assays is easy and it is possible to repeat it as frequent as needed, it is not bothersome for the patients. But to obtain the right clinical assessment, methods should be sensitive and specific. The rules of objective clonal cell measurements are clear, as well as the incorporation of the results to the stage of disease. But the value of the changing level of clonal cells during the observation and therapy is not clear, there is no publication on this topic [2]. The latest EORTC blood classification and blood response criteria for flow cytometry were published in 2018 [2] and vary from the recommendations published in 2011 [2,7,9]. The importance of fluctuations between B0-B1-B2 were not evaluated in the clinical trial, so the value of these changes in unknown [2]. As opposed to 2011, EORTC 2018 proposed the use of cytometry to evaluate the response only in stage B2 and only for erythroderma with the classic image [2,9]. In this case FC has extremely limited use. Progression is confirmed when the level of clonal cells has grown >50%, partial regression when 50% of clonal cells have decreased, complete remission is defined as a lack of clonal cells. The significance of fluctuations in the number clonal cells especially in patch, plaque and tumour MF in stages B2 or B1 is unknown therefore not recommended to evaluate [2].

Materials and Methods

We identified 123 patients with a Mycosis fungoides diagnosis in our database. The MF database has been maintained since 2007 to the present. Cytometry was carried out during staging procedures and during follow up visits, every one-two years or as frequent as clinically needed. 396 FC were done. For evaluation of the blood response criteria EORTC 2018 was used and the result was compared with EORTC 2011. The group of patients who started a new line of therapy due to progression between 2018-2019 were chosen for analysis of the response evaluation made by cytometry and PET/CT scan simultaneously. Our material is presented in 6 tables and 2 charts. 104/123 patients had cytometry at the primary staging, 68/123 were eligible to evaluate the correlation with cytometry with skin status (minimum 2 tests) with comparison between EORTC 2011 and 2018, and 42/123 were chosen to evaluate the response rate in PET/CT in correlation with cytometry. Both examinations were carried out at the time of progression when a new line of therapy was started, and after 3-6 months to evaluate response.

Flow Cytometric Immunophenotyping

The flow cytometric evaluation of MF/SS patients was performed on fresh peripheral blood (PB) samples collected in an EDTA tube and prepared by the use of Becton Dickinson reagents. The antibody panel consisted of a control tube and two assay tubes containing the following antibodies, respectively: I – four-colour Multitest CD3/CD8/CD45/CD4 and II – seven-colour combination of single antibodies: CD2, CD3, CD4, CD7, CD26, CD30 and CD45. All tubes were stained according to the manufacturer's instructions, with minor modifications. At first, particular fluorochrome-conjugated antibodies were added to the 100 µl of PB in assay tubes: I - 20 µl Multitest CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC, II - 5 µl CD2 V450, 15 µl CD3 PerCP-Cy5.5, 5 µl CD4 PE-Cy7, 15 µl CD7 FITC, 15 µl CD26 PE, 5 µl CD30 APC and 5 µl CD45 V500-C. There were no antibodies added to the control tube. After 20 min of incubation with mAbs (in the dark, at room temperature), 2 ml of Lysing Solution was added (next 10 min incubation) in order to lyse the red blood cells. Subsequently, all samples were centrifuged at 300×g for 5 min. The supernatant fluid was discarded, and the cells were washed twice with 2 ml of Cell Wash at 200×g for 5 min. The obtained cell pellet was fixed with 0.5 ml of Cell Fix, collected with BD FACS Canto II flow cytometer and analysed with BD FACS Diva Software (Becton Dickinson). For each measurement, data from 10,000 lymphocytes were acquired. To identify abnormal T cells, the gating strategy similar to that described in Horna et al.,(3,10) was used. At first step, CD3+ T cells population was identified and gated in the first assay tube. Secondly, CD3+/CD4+ and CD3+/CD8+ cells were sub-gated among the CD3+ population in order to calculate the CD4:CD8 ratio value. In the second tube, the abnormal expression of other antigens was analysed and the percentage of CD4+/CD7(-) and CD4+/26(-) cells was determined among the CD3+ cells and total lymphocytes. The degree of peripheral blood involvement by MF/SS cells was staged as B0, B1 and B2 according to Olsen et al.,[4] and Swerdlow et al.[8].

PET/CT

All patients were scanned on a dedicated PET/CT scanner (Biograph MCT20 and 126; Siemens, Germany). The patients had

been fasting for at least 4-6 h and blood glucose levels were required to be less than 10 mmol/L before [18F]FDG injection (4.0-5.0 MBq/kg). Scanning was started from the basal skull to mid-thigh after an uptake time of 50-70 min. CT without intravenous or oral contrast scans were performed using a 128 and 20-slice helical CT with a continuous spiral technique (120 KeV; automatic current regulation adjusted to the thickness and density of each patient's body; section thickness of 5 mm). PET scans were obtained for 1.5 min per frame and were reconstructed using an iterative algorithm (Siemens). The response evaluation in PET/CT was according to criteria published by Wahl[9] and Cheson[5].

Results

The majority of patients were diagnosed in more advanced stages. T2b-T4 constituted 70.2% of the whole group. Despite this, B2 features were rare, only in 14 patients (13.46%). B0 was diagnosed in 48 patients, even in T4. In T2b-T4 B0 constituted 25%. Value of features B 0-1 does not correlate with skin or nodal advancement. B2 was diagnosed in stage T3 and T4. The advancement of all 4 classifications elements (TNMB) in group B2 were consistent. Table 1 presents the stages in the whole group with complete primary staging.

	T1a	**T1b**	**T2a**	**T2b**	**T3**	**T4**	**Total, /%**
Number of Patients	**3**	**8**	**20**	**32**	**30**	**11**	**104/100**
B0	2	6	13	17	9	1	48/46.1
B1	1	2	7	14	14	4	42/40.38
B2	0	0	0	1	7	6	14/13.46
N0/Nx	3	7	9	3	0	0	22/21.15
N1-2	0	1	11	24	19	1	56/53.84
N3	0	0	0	5	11	10	26/25
Number of Patients B1/B2	**1**	**2**	**7**	**15**	**21**	**10**	**56/100**
CD4:CD8>10			2	4	5	10	21/37.5

Table 1: Presents primary staging in the whole group (104 patients)– Flow cytometry and lymph node and skin assessment.

The same analysis of primary staging was carried out separately for a group of 68 patients who were available for analysis of the fluctuation of atypical cells between the primary measurement at diagnosis and subsequently, during therapy for response evaluation. Table 2 present data of primary staging patients, whose data were chosen for FC analysis.

	T1a	T1b	T2a	T2b	T3	T4	Total/%
Number of Patients	1	2	10	24	22	9	68/100
B0	0	0	4	11	6	1	22/32.35
B1	1	2	6	13	7	4	33/48.5
B2	0	0	0	0	9	5	14/20.6
N0/Nx	0	1	3	4	0	0	8/11.8
N1-2	1	1	7	18	13	0	40/58.8
N3	0	0	0	2	9	9	20/29.4
CD4:CD8>10	0	0	0	3	4	9	16/23.5

Table 2: Primary staging of 68 patients, who were chosen for FC analysis (minimum 2 FC).

In all 68 patients complete remission (CR) B2 or B1 was not observed, the number of partial regression (PR) was low, only 10.29%. We did not notice the progression (PD) in FC reports. In the analysed group the level of absolute cell count of atypical cell was stable (SD) (88.23%). In 7 cases with PR (B2, B1) the reduction of the absolute count was above 50%, and correlated with cutaneous regression during therapy. In rest of the analysed group (61 patients) the effect of the therapy in the skin or lymph nodes did not correlate with the FC results. Below we present the data FC changing during therapy according to EORTC 2011.

In FC	T1a	T1b	T2a	T2b	T3	T4	**Total**
CR	0	0	0	0	0	0	**0/68**
PR	0	0	0	0	5 (B2~~B1, B1>50%)	2 (B2~~B1)	**7-68** **10.29%**
SD	1 (B1)	2 (B1, B0)	10 (B0, B1)	24 (B0, B1, B2)	16 (B0, B1)	7 (B1, B0)	**60/68** **88.23%**
PD-not observed even in skin progression	0	0	0	0	0	0	**0**
Relapse	0	0	0	0	0	0	**0**
LCT	0	0	0	0	1	0	**1.47%**

Table 3: Presents the FC evaluation according to EORTC 2011 during treatment and observation (68 patients).

CR-complete remission (B2 to B0 or B1 to B0), PR-partial regression- 50% reduction of absolute count B2 or B1, SD stabilisation-B0, B1, B2 no change <50%, PD progression-increase >50%, R-relapse, LCT-large cell transformation.

According to EORTC 2018, FC can be used only in a small part of treated patients, in our material it is only 13.46% of the whole group, and 20.6% in the evaluated group of 68 patients. PR in this group were correlated with the clinical response as well as the clinical course of the disease.

Complete remission B2 to B0 only	0
Partial response B2 50% reduction absolute count	14-4
Stable disease	9
Progressive count B2 only 50% increase absolute count	0
Relapse-increase from B0, B1 to B2	0
Large cell transformation	1

Table 4: Present the results for group B2. Analysis only for group B2-14 patients/68/123.

The evaluation of the response according EORTC 2018 has limited practical significance. Table 5 show the analysis of the small changes in atypical cell count correlated with the evaluation of the T and N features.

Number of patients	T	Type of infiltration	% of skin infiltration	Median Absolute count before and on evaluation RR	T-response	N-response	Clinical benefit
1	T1a	Patch, Only lesion <10%	5%	14.38-12.50	CR	CR N1-NO-	No symptoms
2	T1b	Patch, plaques, papules <10%	6%	7.95- 9	PR	NO	No symptoms
10	T2a	Patch only >10%	15-20	12.50- 13.3	PR	PR	Noticeable improvement

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24	T2b	Patch, plaques, papules >10%	35-45	B1: 14.6-14.8	PR-14	PR12	Better
				B2: 35-24	SD 5	SD 1	Less itching
					PR 5	PD-6	Second line therapy
22	T3	Tumours	40-60	B1: 12.3-13.8	Pr-16	PR-16	Improvement
				B2: 65-40	SD-4	SD 5	No change
					PD-2	PD 3	Second line therapy
9	T4	Erythroderma	80-100	B1-22.8/19.6	PR-6	PR-6	improvement
				B2: 81/67	PD-3	PD-3	Death

Table 5: Presents the fluctuations of atypical clonal cell count during observation/one line of therapy.

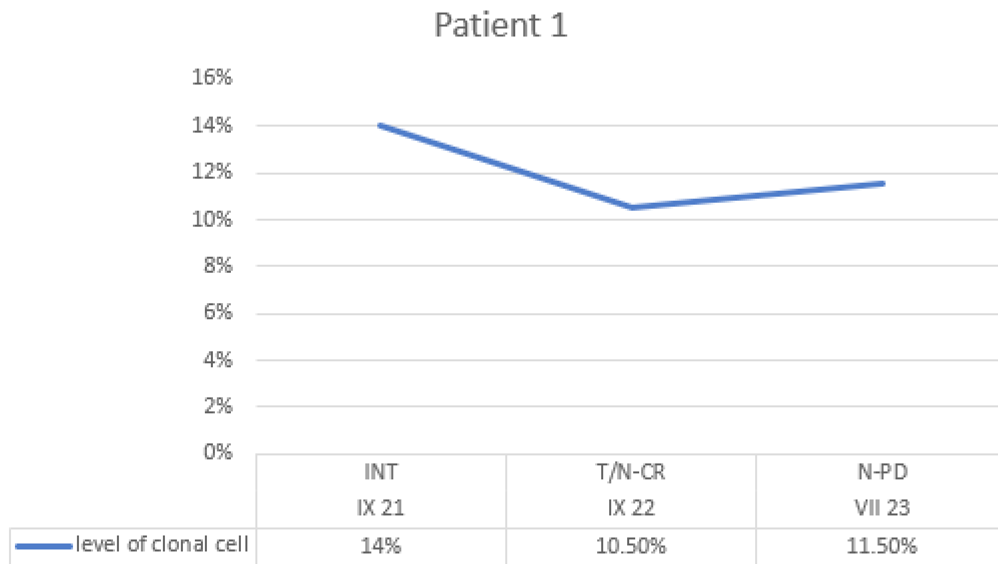
The choice of subsequent lines depends not only on earlier treatment and its response but also it is dependent on the rules of reimbursement at that time. From 2007 to 2011 Interferon alfa 2b, methotrexate and chemotherapy were used, later Bexarotene (from 2011). The peginterferon, Brentuximab vedotin, photophoresis and Anti-PD-1 have been incorporated since 2018. Mogamulizumab was not available in Poland till 2023. During treatment with interferon or methotrexate no clinically significant changes in B1/B2 were observed. Using this "stronger", potentially more efficient drugs was very interesting, if these new ones changed the FC results. Table 6 present the results during different therapies. No changes were observed, even after photophoresis, or stem cell transplantation. Any fluctuations after radiotherapy even if complete remission on the skin were observed. The level of atypical cells remained the same. The strongest effect of therapy was observed in cutaneous lesions, but none in the FC control.

Type of therapy	Brentuximab vedotin	Radiotherapy TSI	Photophoresis	Stem cell transplantation	Anti-PD-1
Number of patients	4	2	2	4	5
Stage at 2 line S/L/F	S: T4-2, T2b-2	S: T4-2	S: T4-2	S: T2b-2, T3-2	S: T4-3,T3-2
	L: N2-3, L1-1	L: N2-2	L-N2- 2	N: N1-3, N2-1	L: N2-5
	F: B0-2, B1-2	F: B1-2	F: B2-2	F: B0-1,B1-3	F: B0-2, B1-2, 2-1

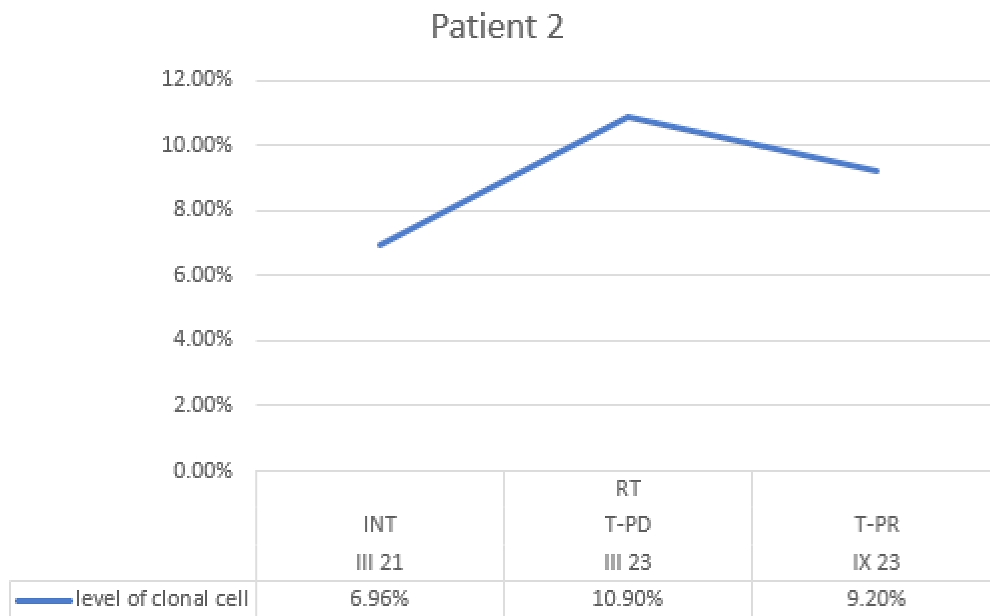
Table 6: Presents FC results during different second line therapies.

Below we present two characteristic types of fluctuations in the absolute cell count during therapy.

Lymph node progression without elevation of absolute atypical cell count (only small fluctuations, clinically not important): Fluctuations between 14% at primary diagnosis to 10% after one year of INT and 12% at presence of iliac lymph nodes.



Diagnosis T1aN1M0B1. INT-interferon was started on IX 21 as first line. One year therapy with complete remission (skin, node), Absolute atypical cell stable. New 2 left iliac nodes confirmed in PET/CT scan, absolute atypical cell stable.



Elevation of absolute atypical blood count in the course of unifocal progression (not clinically important according to EORTC 2018, Progression-according to EORTC 2011).

Diagnosis T1bN0M0 INT-interferon was started on III 2021 with PR in the cutaneous infiltrates on the face. Progression was observed after 2 years of continuous pegINT. Control FC at this time shows some elevation of atypical cells from 6.9% to 10.90%, (PD -EORTC 2011). Progressing lesions were irradiated with complete response, but the level of atypical cells remained elevated, without change until January 2024.

From 2018 PET/CT was incorporated into the primary evaluation stage of the disease as well as for the evaluation response due to better staging of the lymph nodes and better control of the internal organs. We analysed the skin assessment (T value made by a clinician), node assessment (PET/CT) and FC. Even with the criteria form 2011, FC is the most stable value. PET/CT sometimes shows us the cutaneous thickness, but is mainly important for lymph nodes analysis, which are frequently small in diameter but located in different areas. SUV seems to be proportional to skin inflammation. PET/CT examination confirms less CR and PR than was estimated during physical examination.

	Initial diagnosis (number of patients)	Complete response	Partial regression	Progression
PET/CT N+ **Total**	34	8 **8/42-19%**	22 **22/42-52.38%**	4 **4/42-9.52%**
FC (2011) B0 B1 B2 **Total**	15 21 8	0 n.a B0-0 B1/B0-0 **0/42**	n.a B1-2 50% less B2- 2 to B1 **4/42-9.52%**	LCT-1 0 0 0 **1/42-2.38%**
Physical examination <10% >10% >80% Tumours **Total**	2 23 12 5	1 6 3 2 **12/42-28.57%**	1 15 7 3 **26/42-61.90%**	**2** **2** **0** **4/42-9.52%**

Table 7: Response evaluation (42 patients)-one line of therapy between 2018-2020.

The evaluation of the response, first of all in lymph nodes, by PET/CT seems to be very useful and more objective than a clinical assessment. PET/CT scans documented response evaluation are presented below (patient 1: staging, 1 response, progression and second response, and patient 2 diagnosis and response on therapy).

According to EORTC 2018, flow cytometry cannot be a tool for monitoring the response rate. The B2 feature is rare, in our material only 14/123 patients. FC mainly determine the primary stage of the disease. According to criteria published in 2011 a small number of partial regressions were noticed (10.69%) in B1 or B2 patients. The group is not representative to confirm any clinical implications with this finding.

Discussion

The evaluation of responses during treatment of Mycosis fungoides remains difficult. We do not have methods for skin thickness measurement, which seems to be important for the estimation of tumour volume. The features of T rated by type of infiltrate and percent of occupied area is not always objective, T cannot be precisely measured. Physical examination and PET/CT make lymph nodes (LN) assessment more precise. Nevertheless, due to availability and cost, Xray/nuclear isotopes are not suitable for frequently repeated diagnostic methods. FC is a very important tool in the diagnosis of all type of leukaemia or enlargement peripheral LN of unknown origin. In primary cutaneous lymphomas, mainly MF and Sezary Syndrome FC is also recommended [2,3,5,10,11,12,13,14]. Due to the rarity of these diseases, the significance of B1, changes between B0 to B1, and B1 to B0 is unknown.[2] There is no Polish data concerning the value of FC in the evaluation of responses. EORTC recommendations[2,7] include the analysis of the literature covering blood involvement in MF using flow cytometry that were published recently. Our study is a retrospective study. The data confirm that the majority of patients are B0 and B1, regardless of type and extent of skin infiltration. It confirm that the majority of patients have a stable level of atypical cells independently of therapy and the clinical course of the disease. There is no correlation between the features of B with skin and nodal response or progression. The cells CD4+CD7- and CD4+CD26- are confirmatory for diagnosis but not significant in the clinical course and response rate, their presence or not, their amount and their fluctuation. Stable levels of the absolute count of atypical cells, independent of lymph nodes and skin responses, is difficult to explain. The last published data by N. Lewis demonstrates the possibility of the better identification of Sezary cells in FC with a different marker PD-1/CD 279.[15]

Recently, alongside FC, there has been increasing emphasis on the significant role of PCR or high-throughput sequencing in characterizing T-cell receptor (TCR) gene rearrangements for peripheral blood assessment [2,16]. The monoclonal TCR gene rearrangement in skin has been associated with lower overall survival [17] and a shorter time to systemic treatment (TTST) in patients with early-stage MF [18]. However, the limitations of those studies include a lack of skin and peripheral blood TCR clonality comparison, which seems to have a crucial prognostic value. Currently, it is emphasized that only an identical T-cell clone in the skin and blood is relevant and has prognostic value [4,19]. Agar et al., demonstrated that the presence of an identical T-cell clone in blood and skin was associated with significantly lower overall survival, even in B0 patients [19]. Similarly, other studies reported that only patients with the presence of the same most frequently detected clone both in skin and blood had a worse response, worse prognosis,[20,21,22] and a shorter time to TTST compared to patients with different clones [22]. Moreover, recently de Masson et al., reported that tumour clone frequency >25% in the skin assessed with high-throughput sequencing is a strong predictor of aggressive early-stage MF [23,24]. It seems that assessing both FC and TCR gene clones may provide more accurate staging and prognosis of disease than FC alone. However, it should be noted that both FC and TCR clonality using PCR are not routinely performed in early MF stages, not to mention high throughput sequencing, a highly effective method in T-cell clone identification, which remains expensive and available only in highly specialized centres. A limitation of our study is the lack of the comparison of clonality between blood and skin, which does not allow us to explain the stable level of absolute clonal cell count, regardless of treatment, and independent of lymph nodes and skin response. Stable levels of atypical cells may confirm, perhaps, that drugs and methods used for therapy are not fully effective. Another question is if the B0 group should be a different subtype of MF? But the clinical course of patients with subtypes B0 and B1 is the same. The features T and N correlate with each other, but do not correlate with levels of atypical cells. This lack of corelation is observed not only in the clinical course, but also in primary diagnosis. B0 also occurs in T3/4 stage. We tried to verify, in real world data, the statement made in J Scarisbrick's analysis [2] that fluctuation between B0 and B1 are not clinically relevant. The B0, B1 results cannot be an indicator for treatment changes [2]. Our findings are compatible with published data. We observed comparable proportions in B0/B1/B2 in advanced T3 and T4 patients, we confirmed any clinical implications of minor blood involvement and that the fluctuation B0/B1 cannot be included in treatment response evaluation. B2 was rarely observed in our material, complete remission was not observed, but in 4 patients with partial regression the correlation between partial regression of skin and nodal was noticed

Conclusions

Flow Cytometry determines the primary stage of the disease but cannot be a tool for monitoring response rates according to EORTC 2011 or 2018. The fluctuation of clonal cells in a majority of patients does not correlate with the type of response in skin or in lymph nodes. The stable level of clonal cells dominates during the years of patients monitoring. PET/CT scans correlate with clinical evaluation of skin response and seems to be more objective in lymph nodes evaluation than physical examination.

Author Contributions

Conceptualization Ewa Chmielowska. Methodology Ewa Chmielowska, Ewelina Łukasiewicz, Bogdan Małkowski, validation, ECH, EŁ, MSW, BO; formal analysis, ECH, MSW, BO; investigation, ECH; resources, ECH, EŁ, BM.; data curation, ECH, EŁ; writing—original draft preparation ECH; writing—review and editing, ECH, MSW, BO; visualization, ECH; supervision, MSW. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable. The analysis form the hospital database.

Data Availability Statement: The research data are archived in the hospital database, and are or where unavailable due to privacy.

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Conflicts of Interest: The authors declare no conflicts of interest.

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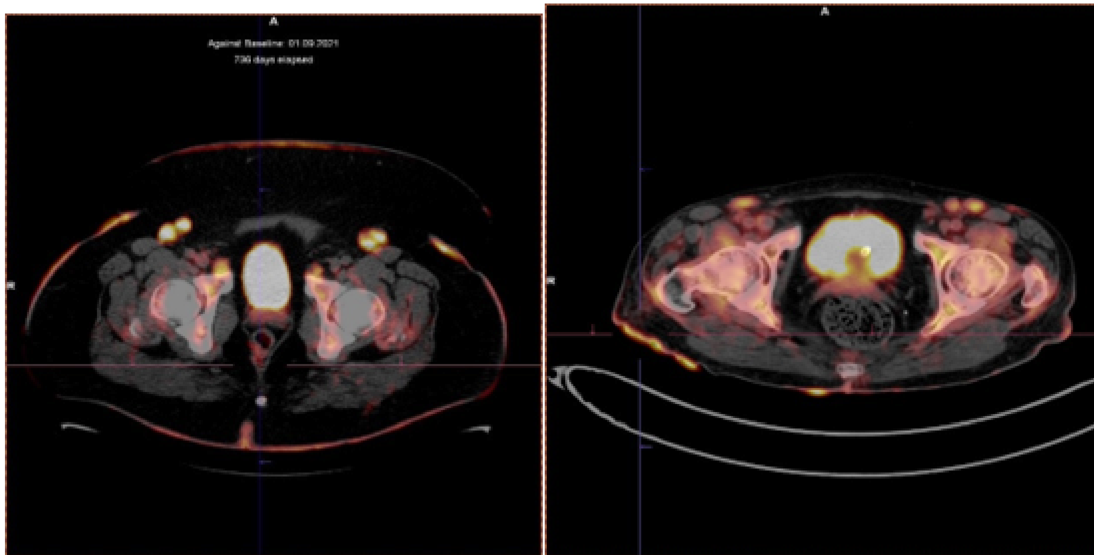
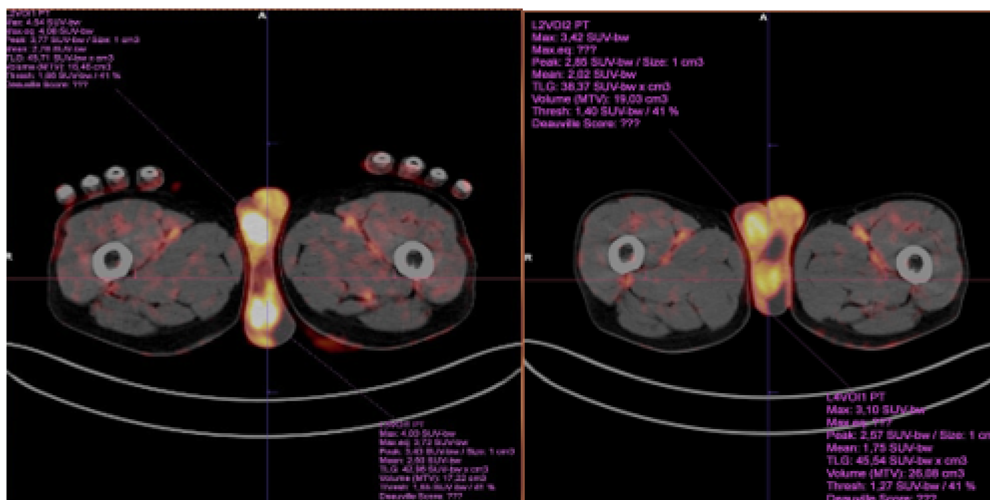
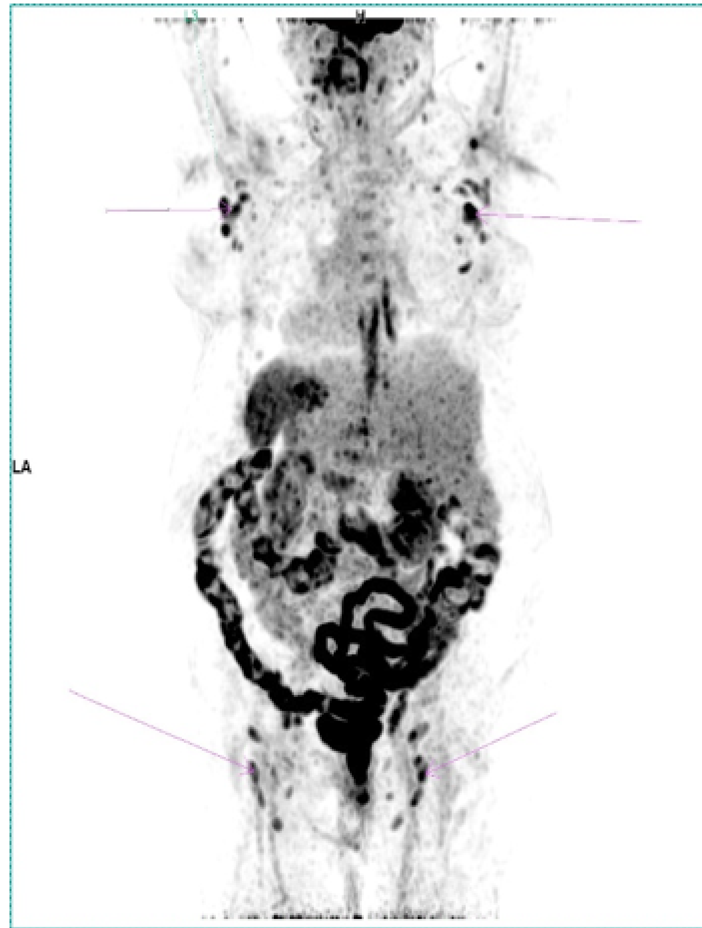
Appendix A



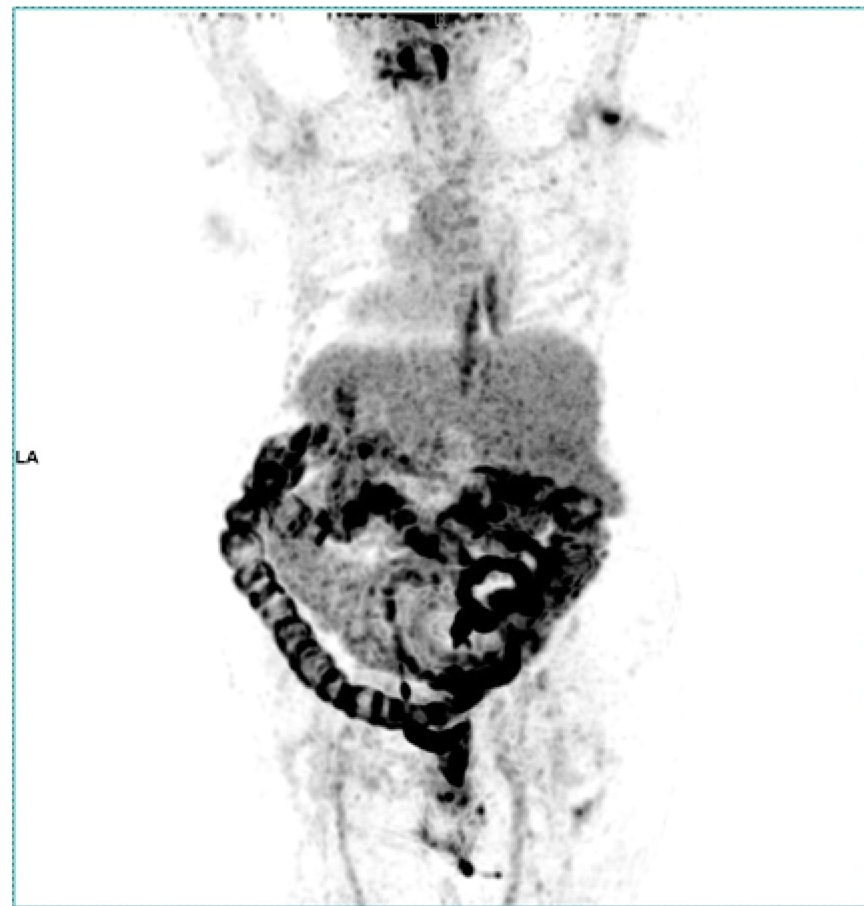
Patient 1 and 2 – skin infiltration and active inguinal lymph nodes – primary diagnosis.



Patient 1-Skin of Scrotal infiltration-Partial regression during therapy primary diagnosis.



Patient nr 3 Before brentuximab vedotin- enlarged lymph nodes.



Patient nr 3 After brentuximab vedotin- remission in lymph nodes.

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