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ORIGINAL ARTICLE – CANCER RESEARCH

The vulvar immunohistochemical panel (VIP) project: molecular profiles of vulvar Paget's disease

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Abstract

Purpose To investigate the expression of biological markers in primary vulvar Paget's disease (VPD).

Methods Forty-one patients referred to a single major Center for Gynecologic Oncology from January 2008 to June 2018 were enrolled retrospectively: 30 non-invasive-VPD and 11 invasive-VPD. A total number of 60 samples, from all the 41 vulvar sites (VS), 8 metastatic lymph node sites (MLS) and 11 successive recurrent disease in vulvar site (RVS), were tested for an immunohistochemical panel, including the following markers: PD-L1, CD3, MSH2, MSH6, MLH1, PMS2, HER2/neu, EGFR, p16, p53, Ki67, ER, PR, AR, VEGF and CD31.

Results We found a positive PD-L1 in 10% of non-invasive-VPD and 27% of invasive-VPD (18% VS; 38% MLS). ER and AR were expressed respectively in more than 70% and 75% of all specimens. HER2/neu amplification was found in 21% of non-invasive-VPD and 45% of invasive-VPD (40% VS; 38% MLS). A machine learning cluster analysis identified three groups among non- invasive-VPD: cluster-1 with higher median ER expression (40%); cluster-3 with more frequent HER2/neu overexpression (46%). Among invasive-VPD, two clusters were found: the second with more frequent HER2/neu overexpression (67% vs. 0%) and nodal metastases (100% vs. 25%). Repeating the IHC panel on the correspondent MLS and RVS, it significantly changed, respectively, in 50% and 27%.

Conclusions This study reveals the expression of PDL-1 and ER and confirms the expression of HER2/AR in VPD; new bases are provided to design multicenter clinical trials on personalized target therapies.

Keywords Gynecological cancers · Biostatistics · Molecular targets · Vulvar cancer · Immunohistochemistry

Giorgia Garganese and Frediano Inzani have contributed equally to the article and share the first authorship.

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Introduction

Vulvar Paget's disease (VPD) is historically classified in primary/cutaneous (type 1) and secondary/non-cutaneous, arising from malignancies of other districts (type 2 from rectal/anal adenocarcinoma, type 3 from urogenital adenocarcinoma) (Wilkinson and Brown 2002). Type 1 is mainly a non-invasive adenocarcinoma; more rarely it invades deeply (> 1 mm) to the reticular dermis or subcutaneous tissue. No guidelines are available for its management and there is no scientific evidence that one treatment is superior to others (Edey et al. 2013).

In non-invasive-VPD, prognosis is very favorable and surgery remains the mainstay of treatment. Anyway, it is difficult to obtain microscopic free surgical margins; moreover, local recurrence rates after surgery vary from 34 to 56% and patients often experience a real "surgical calvary" (van der Linden et al. 2016).

Conversely, invasive-VPD still has a merciless prognosis despite all combined local and systemic therapies. Local treatments include conservative and radical surgery, often supported by reconstructive surgery, to repair large defects and prevent lymphatic side effects; they also include radical or adjuvant radiotherapy, each provided both on the primary vulvar site and metastatic lymph nodes (Gentileschi et al. 2016; Van der Zee et al. 2008; Garganese et al. 2017; Gentileschi et al. 2017; Tagliaferri et al. 2018). Moreover, systemic therapies mostly consist in non-standardized schedules, "borrowed" from other adenocarcinomas and frequently non-effective.

With the aim of expanding the knowledge of this rare disease, in the present study we focused on four main pathways, known to influence carcinogenesis: tumoral immune microenvironment, activation of oncogenic growth factor-receptors, hormonal environment and neo-angiogenesis (Mantovani et al. 2019).

In detail, starting from a series of VPD-patients, our primary aim was to investigate the immunohistochemical (IHC) expression of biological markers that could serve as potential prognostic/therapeutic factors, including PD-L1, CD3, MSH2, MSH6, MLH1, PMS2, HER2/neu, EGFR, p16, p53, Ki67, ER, PR, AR, VEGF and CD31. Secondarily, based on the IHC results, we clusterized patients according to their molecular profiles investigating the correlation between the clusters and the clinical, histopathological and outcome features.

Materials and methods

Patients

The present study, approved by the Institutional Ethics Committee (Prot. 6996/18. ID: 1932), included all the consecutive patients with a histologically confirmed diagnosis of primary VPD, referred to the Gynecologic Oncology of our Center (Fondazione Policlinico Universitario Agostino Gemelli, IRCCS—Rome, Italy) between January 2008 and June 2018. Patients signed a written consent to data collection and to the use of personal records for health research.

The flow chart of the study is reported in Fig. 1.

Sample processing

Samples from the vulvar sites (VS) of all the 41 patients enrolled were tested for the IHC panel, that was repeated on samples from eight metastatic lymph node sites (MLS) and finally on samples from the vulvar site of all the 11 successive recurrences (RVS).

Haematoxylin and eosin stained sections were reviewed by four pathologists. Subsequently, the corresponding paraffin blocks were cut using a rotary microtome to obtain slices with a thickness of 3 µm. The following antibodies were used: anti-ER SP1, anti-PR 1E2, anti-Ki67 30-9, anti-HER2/neu, anti-p53 Bp53-11, anti-p16 CINtec/Histology, anti-CD3 2GV6, anti-MLH1 M1, anti-MSH2 G219-1129, anti-MSH6 44, anti-PMS2 EPR3947, anti-PD-L1 SP263 (all from ROCHE/VENTANA, prediluted); anti-CD31 JC70A (LEICA, prediluted); anti-VEGF VG1 and anti-EGFR E30 (DAKO/AGILENT, 1:50); anti-AR AR441 (DAKO/AGILENT, 1:100). Silver in situ hybridization (SISH) was used to better define HER2/neu status. The status of HER2/neu gene was a function of the ratio between the number of copies of the gene and the number of copies of chromosome 17 (Chr17) for each cell.

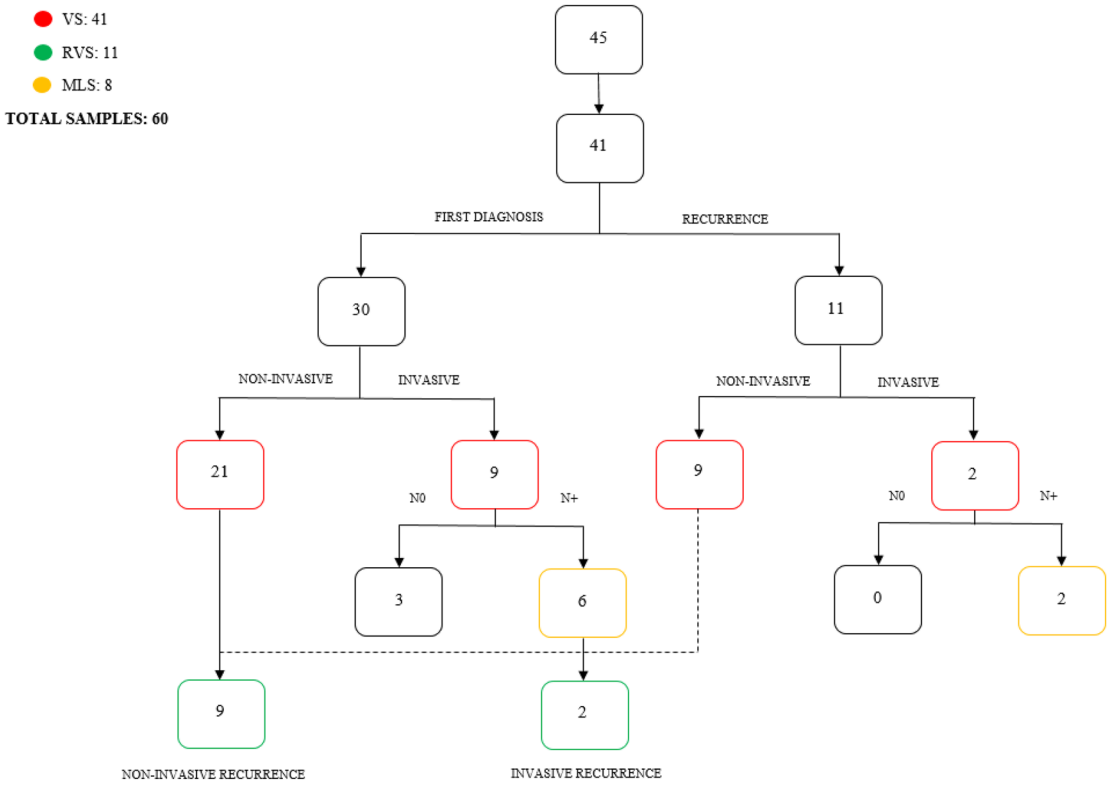
Immunohistochemical interpretation

Tumoral cells showing membrane staining for PD-L1 were regarded as positive, without considering intensity. We used placental tissue as a positive control. A tumor site was defined "PD-L1 positive" if at least 5% of Paget cells were positive. Lymphocyte T marker CD3 was used to evaluate immune infiltrate density, in all vulvar sites, and distribution, only in invasive ones. The expression of MSI markers MSH2, MSH6, MLH1 and PMS2 was considered maintained when present at a nuclear level, regardless of intensity.

For HER2/neu expression, membrane staining was evaluated according to the ASCO-CAP guidelines described for breast cancer (Wolff et al. 2013).

EGFR was assessed on the base of the intensity of membrane staining compared to the surrounding non-neoplastic epithelium.

For p16 expression, diffuse weak staining was considered negative; nuclear and cytoplasmic expression, of variable intensity, in less than 50% of tumor cells, was defined "patchy"; diffuse nuclear positivity of tumor cells, with at



VS vulvar sites, MLS metastatic lymph node sites, RVS recurrent vulvar sites, N0 node negative, N+ node positive.

Fig. 1 Flow-chart of the study. *VS* vulvar sites, *MLS* metastatic lymph node sites, *RVS* recurrent vulvar sites, N0 node negative, N + node positive

least moderate intensity, was considered as overexpression. Nuclear overexpression of p53 compared to negative cases (wild type) was considered to be such when present markedly (in > 75% of tumor cells) at the nuclear level. Ki67 was expressed as the average percentage of positive nuclei, regardless of intensity.

A tumor showing ER, PR or AR nuclear staining in a fraction of neoplastic cells ≥ 1% was considered positive.

VEGF was evaluated on the base of the intensity of cytoplasmic staining compared to the endothelium, and defined as negative, weak or intense. Micro vessel density (MVD) was only studied in the primary tumor site with CD31. In each slide, three areas with an increased number of capillaries and small venules were identified in the dermis below the neoplasm, at low magnification. In these three areas, maximum MVD and mean MVD were assessed with a high magnification field count (HPF 400x). The MVD was also studied in the non-neoplastic surrounding tissue (ST) for comparison.

Statistical analysis

To analyze differences and similarities between patients and between variables, we applied the functions of the "CluMix" package through the R analysis software (3.4.4 version) (Hummel et al. 2017). These allowed us to produce graphic representations of the clusterization process, in the form of dendrograms and heatmaps. For comparisons between more than two groups/clusters, the statistical significance of continuous variables was analyzed in GraphPad 5.0 through the classic one-way Anova procedure, followed by post-tests corrections for multiple comparisons. For comparisons between only two groups, instead, the non-parametric Mann–Whitney *t* test was used.

With regard to OS and DFS, statistical analyses were performed using GraphPad 5.0 software; Kaplan–Meier survival curves were created and compared with each other through the log-rank test. Statistical significance was considered achieved if the p value obtained was lower than 0.05.

Results

Patients characteristics

30/41 patients (73%) had a non-invasive-VPD (21 primary and nine recurrent disease). Eleven (27%) had an invasive-VPD (nine primary and two recurrent disease) and eight

showed lymph node metastases (inguinal a/o pelvic a/o paraaortic). Patients' clinical characteristics are summarized in Table 1.

Median FUP after treatment was 16.3 months (range 0–111 months). During FUP, overall 11 patients recurred in the vulvar site and four patients progressed to systemic disease before 6 months. Median time to first recurrence/progression was 16 months.

Immunohistochemistry

Tumoral immune microenvironment

The main results of IHC staining are summarized in Table 2. We found PD-L1 positive tumors in 3/30 (10%) non-invasive-VS and 2/11 (18%) invasive-VS, with no significant difference. Moreover, 3/8 (38%) MLS were PD-L1 positive. Overall, 27% of patients affected by invasive disease had a PD-L1 positive tumor in the VS and/or MLS.

The intensity of immune infiltrate did not differ significantly between non-invasive and invasive-VS; interestingly, with regard to distribution, 70% of invasive-VS showed, in addition to a peritumoral infiltration, also an intratumoral one. Furthermore, all PD-L1 positive sites belonged to this group.

In our analysis, all tested samples showed retained immune-expression of mismatch repair related proteins (MSH2, MSH6, MLH1 and PMS2), therefore, these markers were excluded from all subsequent correlation analysis.

Hormonal environment

The expression of ER and AR was found, respectively, in at least 70% and 75% of all specimens from VS and MLS; conversely, PR was expressed in about 20% of all specimens, including MLS; for all of them, there was no significant difference between non-invasive and invasive-VS.

Oncogenic growth factor-receptors

HER2/neu amplification was found in 6/30 (21%) of non-invasive-VS (one missing data), 4/11(40%) of invasive-VS (one missing data) and 3/8 (37.5%) of MLS. Overall, 45% of patients affected by invasive-VPD showed HER2/neu amplification in VS and/or MLS.

We observed EGFR high expression in in one VS and in one MLS, respectively.

The p16 expression showed a "patchy" pattern prevalently in the VS, respectively, in 14/30 (47%) non-invasive-VS and 6/11 (55%) invasive-VS (no significant difference), and a "diffuse and weak" pattern in 7/8 (88%) MLS.

Table 1 Patients' clinical features

Total *n*	41
Age	
Median ($q_{1/4}$–$q_{3/4}$) [range]	67 (62–73) [43–88]
BMI[a]	
Median ($q_{1/4}$–$q_{3/4}$)	25.7 (23.2–30.4)
Comorbidities[b]	
None	13 (33.3)
CV	20 (51.3)
Hypertension	
Cardiac arrhythmia/ischemia	
Cerebrovascular disease	
Metabolic	7 (17.9)
DM/Impaired glucose tolerance	
Hypercholesterolemia	
Autoimmune thyroid disease	8 (20.5)
Smoke[c]	
Smoker/Ex-smoker	11 (45.8)
Non-smoker	13 (54.2)
Personal history of cancer[b]	
Synchronous/previous malignant tumor	8 (20.5)
Breast Cancer	5 (12.8)
Endometrial Cancer	1 (2.6)
Lung carcinoid tumor	1 (2.6)
Basal cutaneous cell carcinoma	1 (2.6)
Personal gynecological history	
Menopausal status	
Pre-menopause	3 (7.3)
Post-menopause	38 (92.7)
Previous pregnancies[d]	2 [0–6]
Previous births[d]	2 [0–4]
Category	
First diagnosis	30 (73.1)
Recurrence	11 (26.9)
Number of previous recurrences	
Median [range]	0 [0–4]
Previous treatment	
None	27 (65.9)
Surgery	9 (22.0)
Radiotherapy	3 (7.3)
Chemotherapy	0 (0)
Imiquimod	2 (4.9)
Photodynamic therapy	1 (2.4)
Other medical treatments	2 (4.9)

Data are *n* (%) or median ($q_{1/4}$–$q_{3/4}$) [range], as appropriate. Because of rounding, percentages do not always sum to 100%

BMI body mass index, *CV* cardiovascular, *DM* diabetes mellitus

[a]Data available for 32 patients

[b]Data available for 39 patients

[c]Data available for 24 patients

[d]Data available for 36 patients

Table 2 Immunohistochemical panel of vulvar site and lymph node metastasis

	NI-VS $n = 30$	I-VPD	
		I-VS $n = 11$	MLS $n = 8$
PD-L1 (positive cells %)			
Median ($q_{1/4}$–$q_{3/4}$) [range]	0 (0–0) [0–10]	0 (0–3) [0–10]	0 (0–3) [0–20]
Positive (≥ 5%)	3 (10.0)	2 (18.2)	3 (37.5)
Negative	27 (90.0)	9 (81.8)	5 (62.5)
MSH2			
Positive	30 (100)	11 (100)	8 (100)
Negative	0 (0)	0 (0)	0 (0)
MSH6			
Positive	30 (100)	11 (100)	8 (100)
Negative	0 (0)	0 (0)	0 (0)
MLH1			
Positive	30 (100)	11 (100)	8 (100)
Negative	0 (0)	0 (0)	0 (0)
PMS2			
Positive	30 (100)	11 (100)	8 (100)
Negative	0 (0)	0 (0)	0 (0)
ER (positive cells %)			
Median ($q_{1/4}$–$q_{3/4}$)	23 (0–51)	10 (3–55)	18 (2–35)
Positive (≥ 1%)	21 (70.0)	8 (72.7)	6 (75.0)
Negative	9 (30.0)	3 (27.3)	2 (25.0)
PR (positive cells %)			
Median [range]	0 [0–50]	0 [0–15]	0 [0–1]
Positive (≥ 1%)	7 (23.3)	2 (18.2)	2 (25.0)
Negative	23 (76.7)	9 (81.8)	6 (75.0)
AR (positive cells %)			
Median ($q_{1/4}$–$q_{3/4}$) [range]	40 (1–55)	40 (13–68)	40 (14–45)
Positive (≥ 1%)	23 (76.7)	10 (90.9)	7 (87.5)
Negative	7 (23.3)	1 (9.1)	1 (12.5)
HER2/neu[a]			
Negative	23 (79.3)	6 (60.0)	5 (62.5)
Overexpressed/amplified	6 (20.7)	4 (40.0)	3 (37.5)
EGFR			
Low expression	30 (100)	10 (90.9)	7 (87.5)
High expression	0 (0)	1 (9.1)	1 (12.5)
p16			
Weak diffuse	6 (20.0)	4 (36.4)	7 (87.5)
Patchy	14 (46.7)	6 (54.5)	0 (0)
Intense diffuse	10 (33.3)	1 (9.1)	1 (12.5)
p53			
Wild type	24 (80.0)	10 (90.9)	6 (75.0)
Overexpressed	6 (20.0)	1 (9.1)	2 (25.0)
Ki67[2] (average % of positive nuclei)			
Median ($q_{1/4}$–$q_{3/4}$)	–	10 (5–20)	20 (19–34)
Lymphocytic infiltrate (CD3)			
Absent	0 (0)	0 (0)	–
Slight	18 (60.0)	8 (72.7)	–
Moderate	12 (40.0)	3 (27.3)	–
Intense	0 (0)	0 (0)	–

Table 2 (continued)

	NI-VS $n = 30$	I-VPD	
		I-VS $n = 11$	MLS $n = 8$
TILs distribution[b]			
Peritumoral	–	3 (30)	–
Intratumoral	–	0 (0)	–
Both	–	7 (70)	–-
VEGF			
Negative	26 (86.7)	10 (90.9)	5 (62.5)
Weak	1 (3.3)	1 (9.1)	3 (37.5)
Intense	3 (10.0)	0 (0)	0 (0)
MVD			
Mean MVD—median ($q_{1/4}$–$q_{3/4}$)	25 (20–25)	20 (19–21)	–
Max MVD—median ($q_{1/4}$–$q_{3/4}$)	27 (22–35)	26 (23–35)	–
MVD sane tissue—median ($q_{1/4}$–$q_{3/4}$)[c]	13 (12–15)	15 (11–19)	–

Data are median ($q_{1/4}$–$q_{3/4}$) [range] or *n* (%), as appropriate. Because of rounding, percentages do not always sum to 100%, Only EGFR expression achieved a statistically significant difference in the comparison between I-VS and NI-VS

NI-VS non-invasive vulvar site, *I-VS* invasive vulvar site, *MLS* metastatic lymph nodal site, *I-VPD* invasive vulvar Paget's disease, *PD-L1* programmed death-ligand 1, *MSH2* mutS protein homolog 2, *MSH6* mutS protein homolog 6, *MLH1* mutL protein homolog 1, *PMS2* postmeiotic segregation increased 2, *ER* estrogen receptor, *PR* progesterone receptor, *AR* androgen receptor, *HER2/neu* human epidermal growth factor receptor 2, *EGFR* epidermal growth factor receptor, *TILs* tumor infiltrating lymphocytes, *VEGF* vascular endothelial growth factor, *MVD* microvessel density, *ns* non-significant

[a]In two cases HER2/neu 2 + by immunohistochemistry, it was not possible for technical reasons to proceed with SISH (silver in situ hybridization) reaction

[b]Data available for 10/11 I-VS

[c]Data available for 26 cases

p53 resulted overexpressed in 6/30 (20%) non-invasive-VS and in 1/11 (9%) invasive-VS (no significant difference), raising to 2/8 (25%) in MLS.

Median Ki67 was 10% (range 5–20%) in invasive-VS and 20% (range 19–34%) in MLS.

Neo-angiogenesis

Inconsistent results derived from VEGF immunostaining, with only three non-invasive-VS showing intense positivity.

Mean and maximum MVD were similar between non-invasive and invasive-VS (25 vs. 20/HPF and 27 vs. 26/HPF, respectively). Conversely, the ratio between the MVD counted in the neoplastic and non-neoplastic component of each sample (that we called PD/ST ratio) showed a median value of 1.95, meaning an almost double vascularity in the neoplastic tissue.

Cluster analysis

According to the tumor molecular profile, a cluster analysis was performed, dividing patients in two populations on the base of invasiveness that is the main known prognostic factor.

Among the non-invasive-VPD (Fig. 2a), we identified three main clusters, respectively of 13, six and 11 patients: cluster-1 was characterized by a significantly higher median ER expression (40%), a significantly less intense immune infiltrate and a significantly more frequent p16 "patchy" expression (77%) compared to the other two clusters; cluster-2 was characterized by a significantly more frequent p16 pathological overexpression (67%) and by a significantly more frequent p53 overexpression compared to the other two clusters (100%); cluster-3 was characterized by a significantly more frequent HER2/neu amplification (46%) and more intense immune infiltrate (82%) compared to the other two clusters and by a significantly higher median of mean MVD (25/HPF).

Among patients who underwent surgery with free macroscopic disease margins, vulvar recurrences occurred as follows: three (27%) in cluster-1, with a median time to first recurrence of 10 months (range 4–63 months); two (33%) in cluster-2, with a median time to first recurrence of 29 months (range 18–40 months); finally, two (22%) in cluster-3, with a median time to first recurrence of 63 months (range 15–111 months). No patient died of disease.

Among patients with invasive-VPD (Fig. 2b), we identified two main clusters, respectively, of four and seven

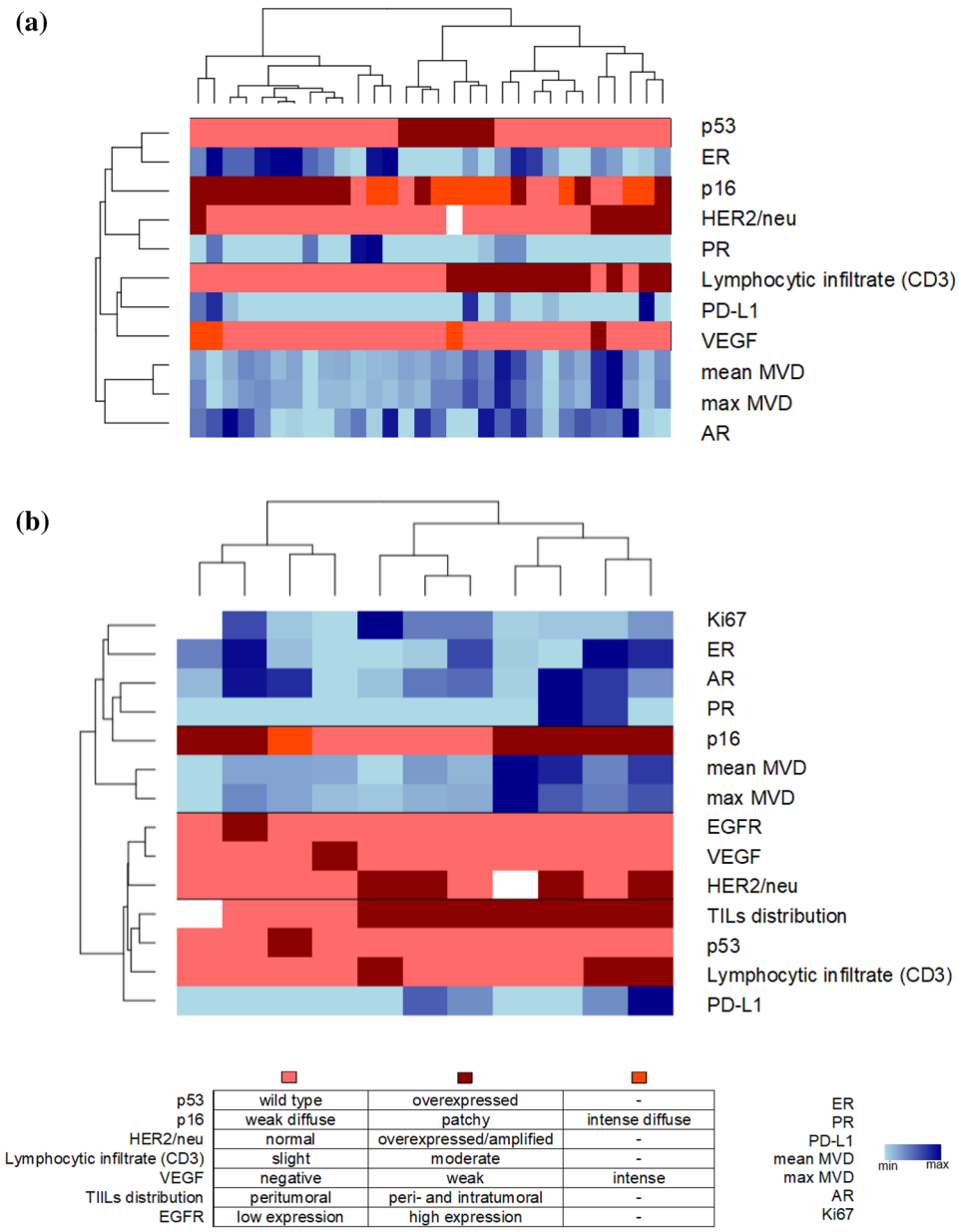


Fig. 2 Cluster analysis: NI-VPD and I-VPD according to molecular profiling of the vulvar site. Dendograms and heatmaps showing the clustering among patients with NI-VPD (non-invasive vulvar Paget's disease, **a**) and I-VPD (invasive vulvar Paget's disease, **b**). The horizontal dendograms give a representation of the hierarchic subdivision in, respectively, three and two main groups of patients (clusters) based on their VIP similarities, globally considered. A maximum distance unit of 0.6 has been chosen on the vertical axes to identify the main clusters. The heatmaps are an illustration of the distribution among clusters of continuous and categorical molecular variables composing the VIP. The vertical dendograms represent the clustering among variables. Ki67 and TILs distribution have been studied only in I-VS. No EGFR "high expression" was founded in NI-VPD, therefore it was excluded. White boxes in the heatmaps represent missing data. NI-VPD non-invasive vulvar Paget's disease, I-VPD invasive vulvar Paget's disease, VIP vulvar immunohistochemical panel, I-VS invasive vulvar sites

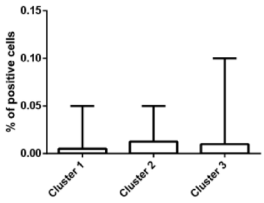
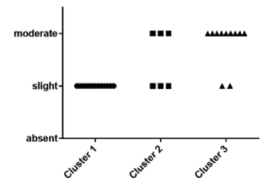
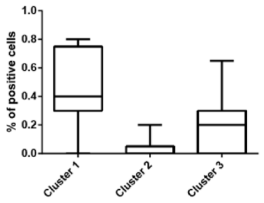
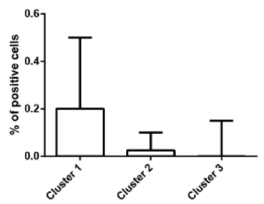
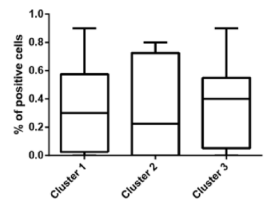
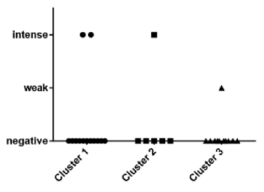
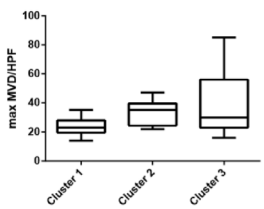
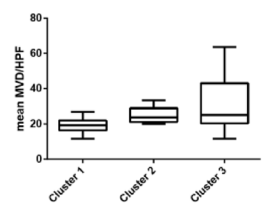
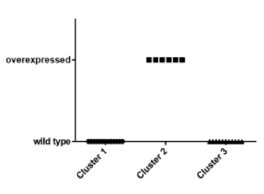
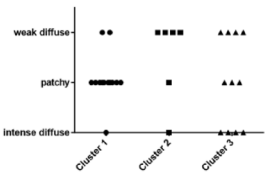
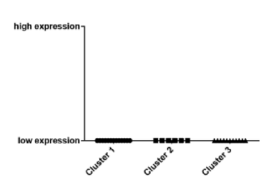
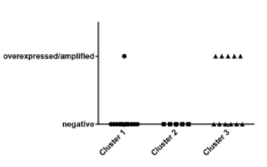
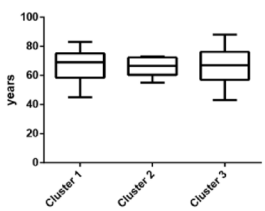
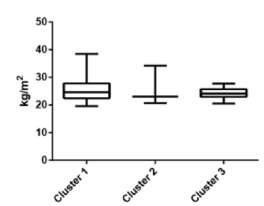
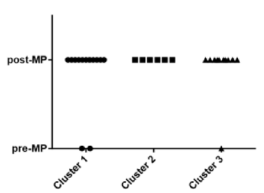
patients. Cluster-2i (invasive), compared to cluster-1i, was characterized by a higher median value of Ki67 (15% vs. 5%). Conversely, there was a significantly more frequent HER2/neu amplification (67% vs. 0%) and a significantly more frequent intra-tumoral immune infiltrate (100% vs. 0%). PD-L1-positive VS were 1/7 in cluster-2i (14% showed PD-L1 expression in at least 5% of Paget cells), while 3/7 (43%) expressed PD-L1 in 1-3% of Paget cells. No VS in cluster-1i exhibited any PD-L1 expression. In cluster-2i there was a higher median depth of invasion (9.5 mm vs. 3 mm), although non-significant, while a significant correlation was found for the presence of nodal metastases (100% vs. 25%). Among patients who completed treatment and were free from macroscopic disease, 60% in cluster-2i experienced metastatic disease progression within 3 months from treatment or had a visceral recurrence within 6 months; moreover, two patients died of disease. To summarize, cluster-2i showed more aggressive biological and histopathological features and a worse outcome.

No significant difference was found among clusters according to clinical features (age, BMI, menopausal status, obstetric history, comorbidities, primary disease/recurrence, previous RT/CT).

A detailed description of cluster features is provided in Tables 3 and 4.

Analyzing the overall patient population by invasion (Fig. 3a), OS only showed a worse trend for invasive disease, while DFS significantly differed among groups

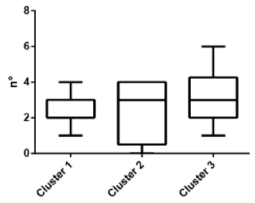
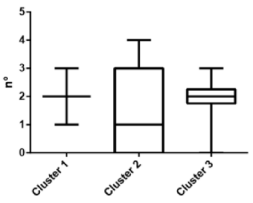
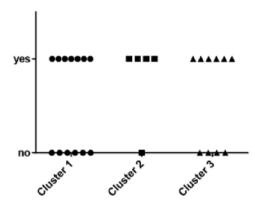
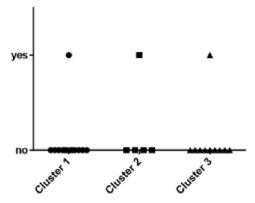
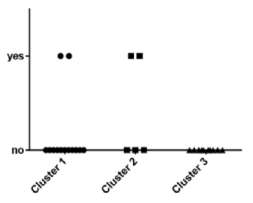
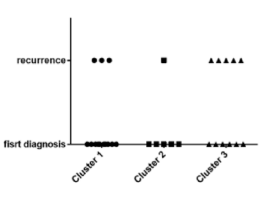
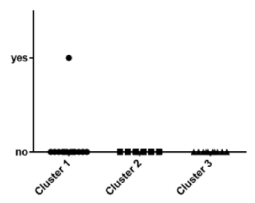
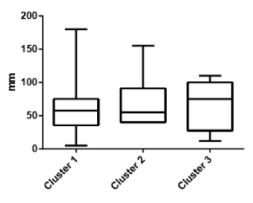
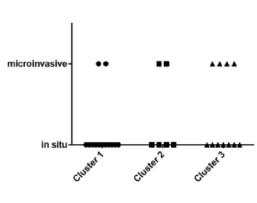
Table 3 Cluster comparison: non-invasive-VPD ($n=13$; $n=6$; $n=11$)

VULVAR IMMUNOHISTOCHEMICAL PANEL										
PD-L1			p-value	Lymphocytic infiltrate			p-value	ER			p-value
			ns ns ns				< 0.001 < 0.001 < 0.05				0.0012 ns ns
0 (0-5)	0 (0-5)	0 (0-10)		0 (0) 13 (100) 0 (0)	0 (0) 3 (50) 3 (50)	0 (0) 2 (18.2) 9 (81.8)		40 (0-80)	0 (0-20)	20 (0-65)	
PR			p-value	AR			p-value	VEGF			p-value
			ns ns ns				ns ns ns				ns < 0.001 < 0.001
0 (0-50)	0 (0-10)	0 (0-15)		30 (0-90)	23 (0-80)	40 (0-90)		11 (84.6) 0 (0) 2 (15.4)	5 (83.3) 0 (0) 1 (16.7)	10 (90.9) 1 (9.1) 0 (0)	
max MVD			p-value	mean MVD			p-value	p53			p-value
			ns ns ns				ns 0.0144 ns				< 0.001 ns < 0.001
23 (14-35)	35 (22-47)	30 (16-85)		19 (12-27)	24 (20-33)	25 (12-64)		13 (100) 0 (0)	0 (0) 6 (100)	11 (100) 0 (0)	
p16			p-value	EGFR			p-value	HER2/neu			p-value
			< 0.001 < 0.001 ns				ns ns ns				ns < 0.001 < 0.05
1 (7.7) 10 (76.9) 2 (15.4)	1 (16.7) 1 (16.7) 4 (66.7)	4 (36.4) 3 (27.3) 4 (36.4)		13 (100) 0 (0)	6 (100) 0 (0)	11 (100) 0 (0)		12 (92.3) 1 (7.7)	5 (100)[1] 0 (0)	6 (54.5) 5 (45.5)	
CLINICAL FEATURES											
Age			p-value	BMI			p-value	Menopausal status			p-value
			ns ns ns				ns ns ns				ns ns ns
69 (45-83)	67 (55-73)	67 (43-88)		25 (20-39)	23 (21-34)	24 (21-28)		2 (15.4) 11 (84.6)	0 (0) 6 (100)	1 (9.1) 10 (90.9)	

($p=0.0128$); evaluating patients on the base of nodal status, the Kaplan–Meier curves (Fig. 3b) of both OS and DFS significantly differed ($p=0.0005$ and $p=0.001$, respectively). Then, comparing the five molecular clusters previously identified (Fig. 3c), neither OS nor DFS resulted in a statistically significant difference.

Finally, we used distance plots to compare directly the global molecular profile of each VS with the correspondent

Table 3 (continued)

CLINICAL FEATURES											
Pregnancies			p-value	Deliveries			p-value	CV comorbidities			p-value
			ns ns ns				ns ns ns				ns ns ns
2 (1-4)	3 (0-4)	3 (1-6)		2 (1-3)	1 (0-4)	2 (0-3)		6 (46.2) 7 (53.8)	1 (20)[1] 4 (80)	4 (40)[2] 6 (60)	
Methabolic comorbidities			p-value	Autoimmune thyreopaties			p-value	Fase of disease			p-value
			ns ns ns				ns ns < 0.001				ns ns ns
12 (92.3) 1 (7.7)	4[1] (80) 1 (20)	9 (90)[2] 1 (10)		11 (84.6) 2 (15.4)	3[1] (60) 2 (40)	10 (100)[2] 0 (0)		10 (76.9 3 (23.1)	5 (83.3) 1 (16.7)	6 (54.5) 5 (45.5)	
				HISTOPATHOLOGICAL FEATURES							
Previuous RT/CT			p-value	Disease extension			p-value	Invasiveness			p-value
			ns ns ns				ns ns ns				ns ns ns
12 (92.3) 1 (7.7)	6 (100) 0 (0)	10 (100)[2] 0 (0)		58 (5-180)	55 (40-155)	75 (12-110)		11 (84.6) 2 (15.4)	4 (66.7) 2 (33.3)	7 (63.6) 4 (36.4)	

Data are median (range) or *n* (%), as appropriate. Because of rounding, percentages do not always sum to 100%. For each variable, *p* values derive from a "two by two" comparison, as follows (beginning from the top): cluster 1 vs. cluster 2; cluster 1 vs. cluster 3; cluster 2 vs. cluster 3

CV cardiovascular, *RT/CT* radiotherapy/chemotherapy

[1]Data available only for 5 patients in cluster 2

[2]Data available only for 10 patients in cluster 3

MLS (Fig. 4a) and RVS (Fig. 4b). The plots are shown as bidimensional matrices with correspondent specimens disposed on the axes. With this model, we found that the IHC panel was significantly different in at least 50% of MLS and 27% of RVS.

Discussion

This is the first study describing PD-L1 expression in vulvar Paget cells. It is evident that this occurs as a mechanism to evade the host's immune response to the tumor for major degrees of aggressiveness, in 10% of non-invasive and almost one-third of invasive forms. We also observed a lymphocytic infiltration within the tumor mass in 70% of invasive cases and all PD-L1 positive tumors belonged to this group. A recent schematization attempted to classify all human cancers into four groups on the basis of the presence or absence of TILs infiltration and PD-L1 expression: both present, both absent or alternatively only one of the two present (Teng et al. 2015). This model, particularly studied in cutaneous melanoma, recognizes the subgroup hypothetically most responsive to immunotherapy as the one showing a simultaneous presence of PD-L1 on tumor cells and TILs within the tumor.

Regarding MSI, unexpectedly no instability was found. MSI in VPD has been reported in a single study by Kang et al. (2016); the authors evaluated IHC expression of proteins encoded by MMR genes, beside also the germinal/

Table 4 Cluster comparison: invasive-VPD ($n=4$; $n=7$)

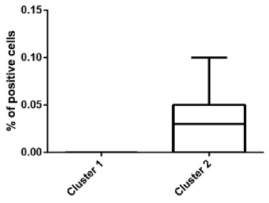
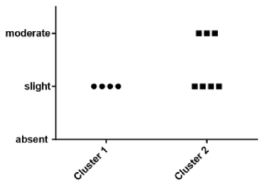
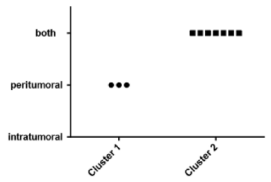
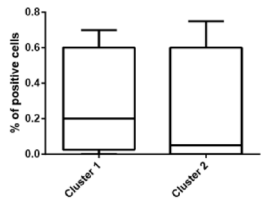
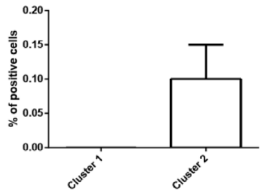
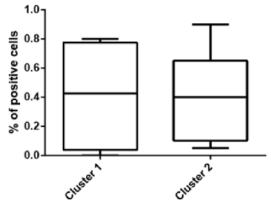
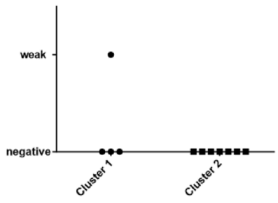
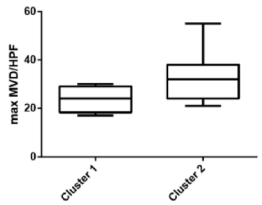
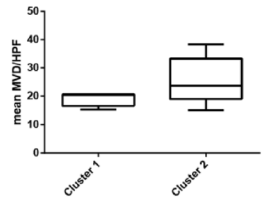
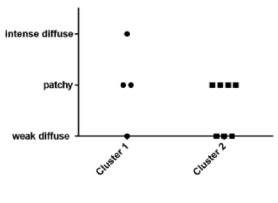
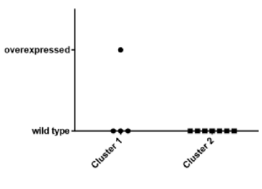
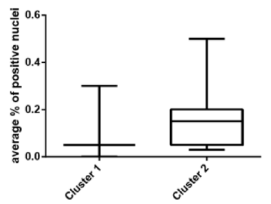
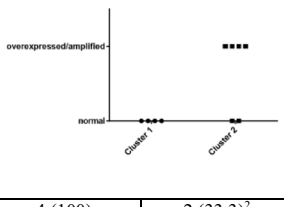
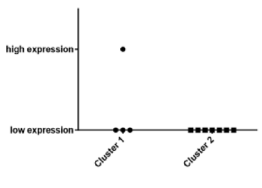
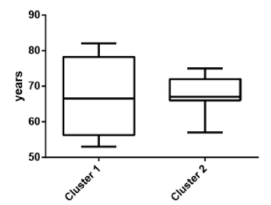
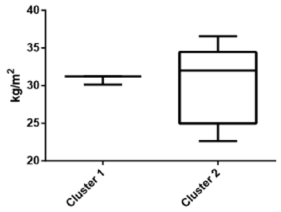
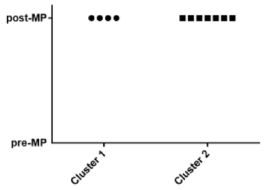
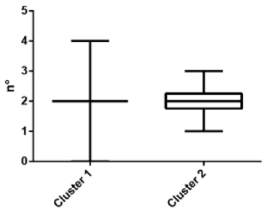
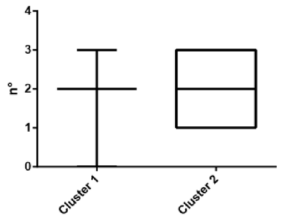
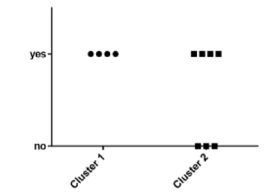
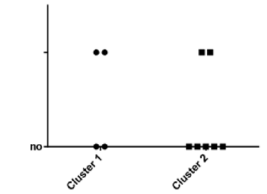
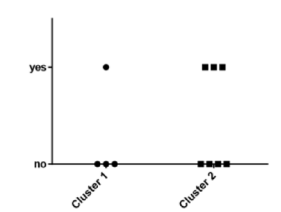
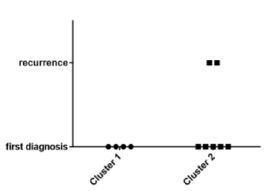
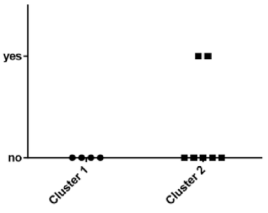
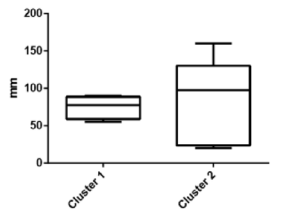
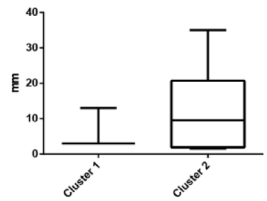
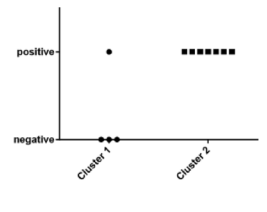
VULVAR IMMUNOHISTOCHEMICAL PANEL							
PD-L1		p-value	Lymphocytic infiltrate		p-value	TILs distribution		p-value
								
0 (0-0)	3 (0-10)	ns	0 (0) 4 (100) 0 (0)	0 (0) 4 (57.1) 3 (42.9)	ns	0 (0) 3 (100)[1] 0 (0)	0 (0) 0 (0) 7 (100)	< 0.001
ER		p-value	PR		p-value	AR		p-value
								
20 (0-70)	5 (0-75)	ns	0 (0-0)	0 (0-15)	ns	43 (0-80)	40 (5-90)	ns
VEGF		p-value	max MVD		p-value	mean MVD		p-value
								
3 (75) 1 (25)	7 (100) 0 (0)	< 0.001	24 (17-30)	32 (21-55)	ns	20 (15-21)	24 (15-38)	ns
p16		p-value	p53		p-value	Ki67		p-value
								
1 (25) 2 (50) 1 (25)	3 (42.9) 4 (57.1) 0 (0)	< 0.001	3 (75) 1 (25)	7 (100) 0 (0)	< 0.001	5 (0-30)	15 (3-50)	ns
						CLINICAL FEATURES		
HER2/neu		p-value	EGFR		p-value	Age		p-value
								
4 (100) 0 (0)	2 (33.3)[2] 4 (66.7)	< 0.001	3 (75) 1 (25)	7 (100) 0 (0)	< 0.001	67 (53-82)	67 (57-75)	ns

Table 4 (continued)

CLINICAL FEATURES								
BMI		p-value	Menopausal status		p-value	Pregnancies		p-value
								
31 (30-31)	32 (23-37)	ns	0 (0) 4 (100)	0 (0) 7 (100)	ns	2 (0-4)	2 (1-3)	ns
Deliveries		p-value	CV comorbidities		p-value	Methabolic comorbidities		p-value
								
2 (0-3)	2 (1-3)	ns	0 (0) 4 (100)	3 (42.9) 4 (57.1)	ns	2 (50) 2 (50)	5 (71.4) 2 (28.6)	ns
Autoimmune thyreopaties		p-value	Fase of disease		p-value	Previuous RT/CT		p-value
								
3 (75) 1 (25)	4 (57.1) 3 (42.9)	ns	4 (100) 0 (0)	5 (71.4) 2 (28.6)	ns	4 (100) 0 (0)	5 (71.4) 2 (28.6)	ns
HISTOPATOLOGICAL FEATURES								
Disease extension		p-value	Depth of invasion		p-value	Lymphnodal status		p-value
								
78 (55-90)	98 (20-160)	ns	3 (3-13)	9.5 (1.5-35.0)	ns	3 (75) 1 (25)	0 (0) 7 (100)	< 0.001

Data are median (range) or *n* (%), as appropriate. Because of rounding, percentages do not always sum to 100%

CV cardiovascular,*RT/CT* radiotherapy/chemotherapy

[1]Data available only for 3 patients in cluster 1

[2]Data available only for 6 patients in cluster 2

somatic gene mutations and MSI through mononucleotide probes.

Our study showed a percentage of ER-positive tumors (at least 70%) that, to our knowledge, has never been reported. We have also described for the first time the ER expression in metastatic lymph node sites. The only three studies that have investigated ER expression in extramammary Paget's disease have found a much lower expression (Diaz de Leon et al. 2000; Liegl et al. 2005; Zhou et al. 2017).

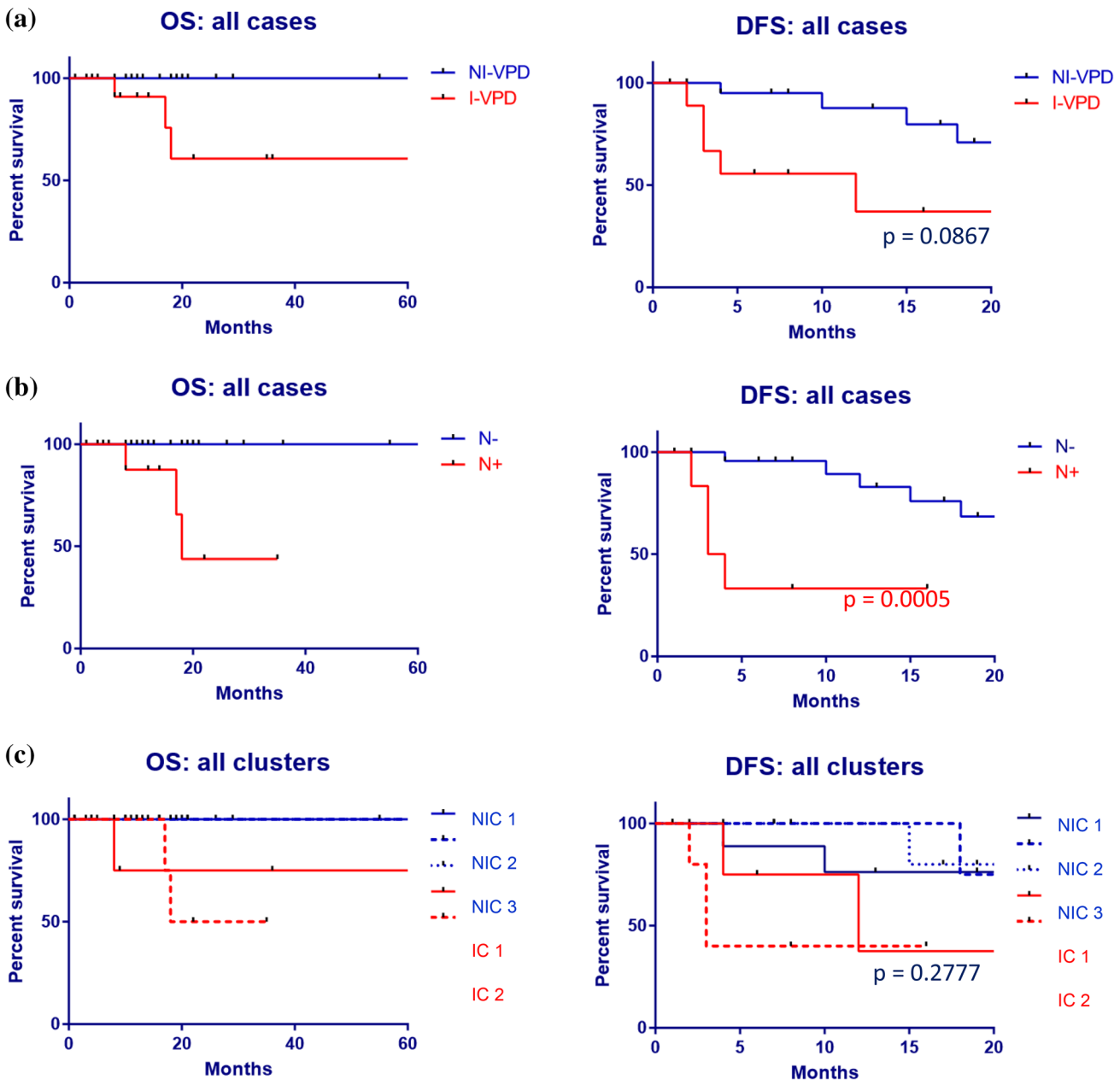


Fig. 3 Overall survival (OS) and disease-free survival (DFS): classic prognostic factors and clusters. Overall survival (OS) and disease-free survival (DFS) curves considering invasiveness (**a**), lymph nodal status (**b**) and identified molecular clusters (**c**). In DFS analysis, six patients were excluded as they were not free from disease at the end of treatment, or were waiting for treatment, or were lost at follow-up prior to treatment. NIC non-invasive cluster, IC invasive cluster

The importance of intratumoral autocrine androgen synthesis for carcinogenesis in VPD disease is rather known: it has been related to invasion and has been described in nodal metastases (Inoguchi et al. 2007; Fujimoto et al. 2000; Azmahani et al. 2015). In our study, only the high prevalence of AR expression in both vulvar and lymph nodal sites has been confirmed. Hence, in our results both ER and AR could be considered widely expressed therapeutic targets for low-cost drugs with a good tolerability profile.

We also confirmed HER2/neu amplification as a fundamental pathogenetic pathway in VPD. This molecular feature is known to be involved in VPD pathogenesis, with a prevalence reaching 40% (Karam et al. 2008), and has been linked to invasion, nodal metastases and recurrences. We observed a higher percentage of amplified tumors among invasive-VS (albeit without statistical significance) and metastases. Overall, in 5/11 patients with invasive disease, HER2/neu was overexpressed/amplified in the primary

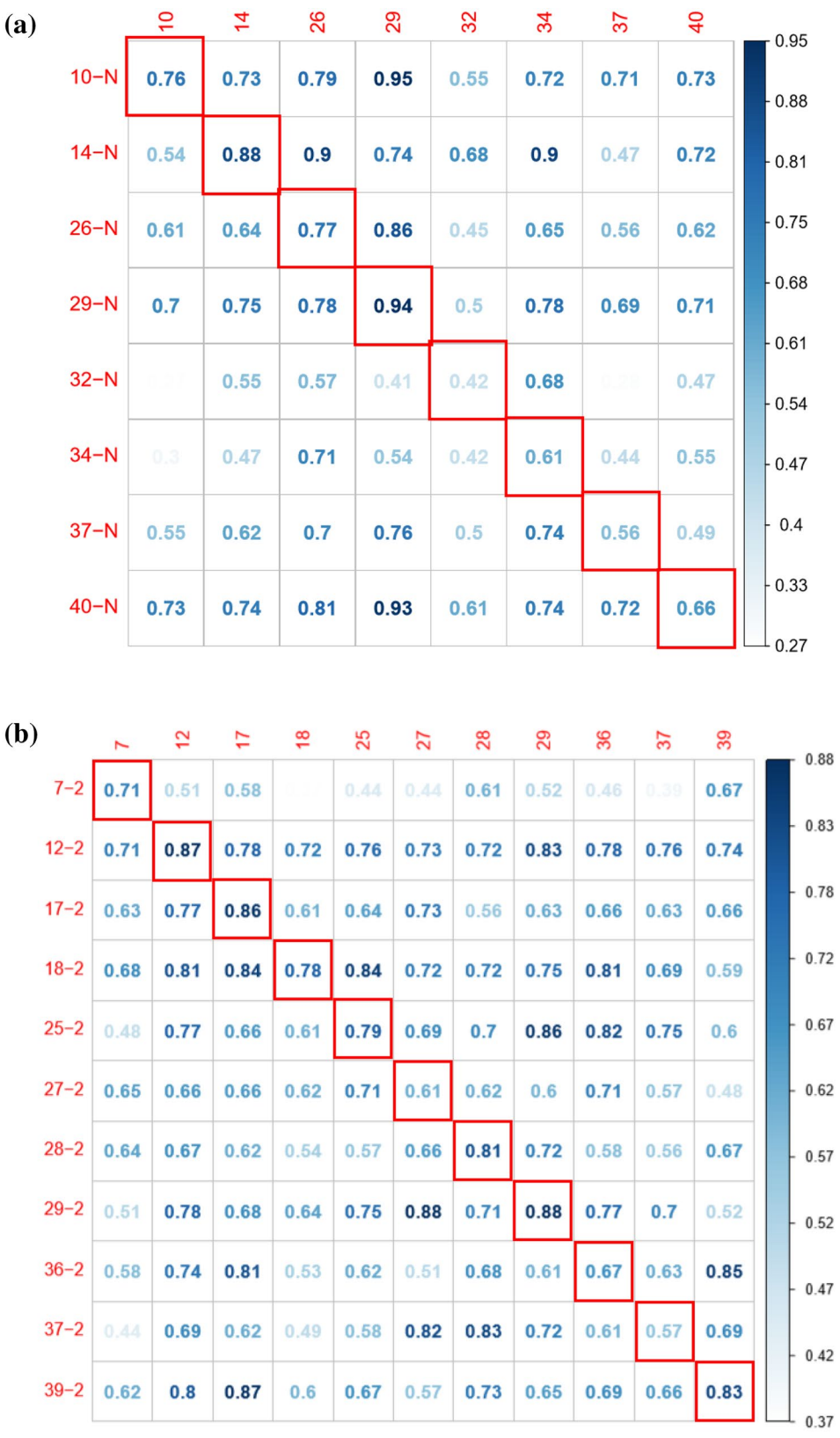


Fig. 4 Correlation plots: the immunohistochemical panels of the vulvar sites compared to the panels of lymph node metastasis (**a**) and local recurrences (**b**). Correlation plots showing global comparison of immunohistochemical panels between vulvar primary tumor and lymphnodal metastasis (**a**) and between vulvar primary tumor and a subsequent vulvar recurrence (**b**) in the same patient. Numbers on the diagonal are a measure of the similarity between statistical units (molecular "fingerprint" of the samples), which we have considered relevant if > 0.7

vulvar tumor and/or in the lymph node metastasis. Thus, almost half of our patients with invasive and/or metastatic disease could have theoretically benefited from therapy with trastuzumab or drugs of the same class.

In our series, we had two cases showing overexpression of EGFR, this has never been described before. One was a vulvar invasive site with a correspondent nodal metastasis where this molecular feature was lost; one was a nodal metastatic site, which also showed HER2/neu amplification both at a vulvar and at a nodal level. In our opinion, these patients would have benefited from sequencing analysis, to identify specific mutations in the EGFR gene amenable for targeted therapy (Larbouret et al. 2012).

We could not derive any definitive conclusion on the prognostic role of p16 overexpression. In small series of the literature, an intense and frequent positivity for p16 in VPD has been described, probably deriving from somatic mutations involving the molecular path Rb-p16 (Al-Obaidy et al. 2018). We did not find a significant correlation of p53 overexpression with invasion, as previously reported in other studies (Ellis et al. 2002a, b). Anyway, p53 was overexpressed in 25% of nodal metastatic sites, and, therefore, seems to be involved in the metastatic potential of the disease, as has already described (Zhang et al. 2003).

VEGF immunostaining in the cytoplasm gave poor results and did not clarify the conflicting results of the previous studies (Ellis et al. 2002a, b; Chen et al. 2008; Hirakawa et al. 2009; Xu et al. 2015). Two studies evaluated MVD in VPD, demonstrating a higher MVD in invasive forms (Ellis et al. 2012; Alessandrini et al. 2018). In our study as well, the extension of tumor vasculature was at least twice the surrounding healthy tissue, as shown by the median PD/ST ratio.

Regarding cluster analysis, among patients with non-invasive tumors, we identified a cluster that relapsed rapidly and that, showing a significant higher ER expression, could potentially benefit from anti-estrogenic maintenance therapy. Among patients with invasive disease, we identified a group with particularly unfavorable prognosis, a trend toward higher Ki67 proliferating index and depth of invasion, and a molecular "fingerprint" that is susceptible to both the use of immunotherapy and anti-HER2 drugs.

Despite the limits of the present study, such as the retrospective design, the small sample size, the heterogeneity of clinical characteristics and treatments delivered and the short median FUP interval, we are sure that some relevant conclusions can be derived, with a direct clinical impact, laying the groundwork for the setting of clinical trials aimed at the use of target therapies.

Further studies are needed to consolidate the obtained results and should be set on a multicenter conception, to allow the inclusion of a sufficient number of cases, to expand the knowledge of this disease.

Author contributions Study conception and design: GG, FI, MF, AF, GS, GFZ, GB. Acquisition of data: GM, GP, AS, MV, SMF, SB, SG, GA. Analysis and interpretation of data: GB, GG, FI, GM, GP, AS, MV, SMF, SB, GA. Drafting of manuscript: GG, FI, GM, GP, AS, MV, SMF, SB, GA. Critical revision: MF, AF, GS, GFZ, SG, GG, FI, GB. Final approval: GG, FI, GM, AS, MV, GB, GP, SMF, SG, SB, MF, AF, GS, GFZ, GA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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