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## Plasmacytoid urothelial carcinoma (UC) are luminal tumors with similar CD8+ Tcell density and PD-L1 protein expression on immune cells as compared to conventional UC

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1 *Original research article*

2 **Plasmacytoid Urothelial Carcinoma (UC) are Luminal Tumors with Similar CD8+ T-**  
3 **cell Density and PD-L1 Protein Expression on Immune Cells as Compared to**  
4 **Conventional UC**

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90 infiltrating lymphocytes

91

92 **Authors' Contributions**

93 Study concept, design: M.K., M.R., J.A. Executed the study: M.K., C.R., J.A., A.D., N.S.  
94 Drafting the article: M.K. Critical revision of the article and approval of the final version  
95 for submission: M.R., J.A., M.S., T.L., Y.A.

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112 **Abstract**

113 Plasmacytoid urothelial carcinoma (UC) is a rare pathological variant of UC with low  
114 chemotherapeutic sensitivity and dismal outcomes. The molecular and immune profiles  
115 of such tumors remain poorly investigated. Herein, we investigated the phenotypical  
116 features of a cohort of plasmacytoid UC (n=32) by comparison to a control group of  
117 conventional high-grade UC with matched clinicopathological characteristics (n=30).  
118 Histopathological analysis included the following antibodies: p63, GATA3, CK5/6, CK20  
119 and HER2. In addition, the density of intra-tumor CD8<sup>+</sup> lymphocytes, and PD-L1  
120 expression in tumor (TC) and immune cells (IC) were evaluated. Plasmacytoid UC  
121 expressed GATA3 (97% vs 86% p=0.18), CK20 (59% vs 36% p=0.08) markers and  
122 showed a significantly higher rate of HER2 overexpression (2+ and 3+ score: 25% vs  
123 0%, p<0.01) compared to controls. A significantly lower expression of CK5/6 (22% vs  
124 56%, p<0.05) and p63 (41% vs 80%, p<0.05) was observed in plasmacytoid UC  
125 compared to controls. The density of tumor-infiltrating CD8<sup>+</sup> cells was similar between  
126 plasmacytoid and conventional UC (p=0.9). PD-L1 expression on IC was similar  
127 compared to conventional UC (p=0.3). Together, our study demonstrated that  
128 plasmacytoid UC belong to the luminal subtype and display a rather inflamed  
129 microenvironment similar to conventional UC. These data support the inclusion of  
130 plasmacytoid variant of UC in clinical trials evaluating immune checkpoint inhibitors  
131 monotherapy or combination immunotherapeutic strategies.

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134       **1. Introduction**

135       Plasmacytoid urothelial carcinoma (UC) is a rare but aggressive variant of bladder  
136       cancer accounting for 2% to 5% of UC in the last 2016 WHO classification [1].  
137       Plasmacytoid UC are distinguished from conventional UC by the presence of  
138       discohesive cells with an abundant eosinophilic cytoplasm and eccentric nucleus, that  
139       resemble plasma cells. Patients often present with an advanced-stage disease, and  
140       common peritoneal carcinomatosis in late-stage disease [2]. Despite conventional  
141       chemotherapy, such patients display dismal outcomes with a high rate of local tumor  
142       relapse and mortality. Loss of E-cadherin expression, due to non-sense mutation of  
143       *CDH1* gene, appears to be common in this variant and may account for the marked  
144       discohesion of these cancer cells [3]. The optimal treatment strategy for this UC variant  
145       is still undefined. The emerging landscape of molecular classification hold clinical  
146       promise in UC [4]. Indeed, the identification of molecularly-defined subgroups may  
147       provide clinically relevant prognostic data to better inform patient care and treatment  
148       planning. However, the issue of how variant morphologies fit into this paradigm has  
149       been unexplored. The tumor immune microenvironment reflects the capabilities of the  
150       tumor to respond or resist to treatment [5]. Programmed cell death ligand 1 (PD-L1)  
151       expression on tumor cells and/or tumor-infiltrating immune cells can modulate  
152       immunosuppression and evasion of host immune system and has been used as a  
153       biomarker to select patients for ICIs. Notably, the PD-L1/ programmed cell death 1 (PD-  
154       1) pathway is a key mediator of local immuno-suppression in the tumor  
155       microenvironment (TME), but can also modulate T cell priming against tumor antigens  
156       in secondary lymphoid tissues [6]. Five immune checkpoint inhibitors (ICIs) targeting

157 PD-1/ PD-L1 have been recently approved as first line therapy in cisplatin ineligible  
158 patients or as second line therapy for patient with advanced and metastatic urothelial  
159 cancer [7]. Importantly, the success of immune checkpoint therapy in advanced UC, as  
160 well as the precedent of neoadjuvant approach toward conventional chemotherapy, has  
161 led to a significant interest in exploring the potential role of neoadjuvant ICIs in muscle  
162 invasive bladder carcinoma. Recently, early-phase clinical trials have reported  
163 promising results of neoadjuvant ICIs alone or in combination before radical cystectomy  
164 in patients with resectable bladder cancer [8].  
165 Herein, the objective of this study was to define for the first time the pathological  
166 immune profile and molecular subtype of plasmacytoid UC.

## 167 **2. Material and Methods**

### 168 *2.1. Patient cohort*

169 After the institutional review board at the Hôpital FOCH approved the study, we  
170 identified 32 cases of plasmacytoid UC in our pathology database from 2002 to 2016.  
171 We retrieved the H&E-stained slides from our archives and two pathologists (C. R. and  
172 M. K) reviewed all the slides. The following criteria were reported: type of surgery (trans-  
173 urethral resection of the tumor or radical cystectomy), percentage of plasmacytoid  
174 variant among tumor cells, association with other histological variants, presence of  
175 carcinoma *in situ*, lympho-vascular invasion and lymph node involvement. Clinical data  
176 from the patient medical records including patient demographics, surgical and medical  
177 treatments, and clinical outcomes were reported in our database. The tumors were  
178 graded according to the World Health Organization/International Society of Urologic

179 Pathology criteria and staged according to the 2010 American Joint Committee on  
180 Cancer TNM criteria. [1] A control group with 30 patients harboring high-grade urothelial  
181 carcinoma (UC), NOS histology was selected. Control cases were matched on the  
182 following criteria: age, sex, and TNM stage. In order to avoid tissue artifacts secondary  
183 to surgical endoscopic resection of the tumor and/or systemic therapy, we carefully  
184 selected treatment-naïve tumor samples providing from the first trans-urethral resection  
185 (TUR) or radical cystectomy specimen.

## 186 *2.2. Immunohistochemistry*

187 Immunohistochemical (IHC) stainings were realized on 4 µm-thick whole tissue sections  
188 from tumor surgical resections. The following antibodies were used: anti-CK20 (clone  
189 M7019, 1:100 dilution, Dako), anti-GATA3 (clone PM405AA, prediluted, Bio Care), anti-  
190 CK5/6 (clone MSK03405, 1:100 dilution Zyomed), anti-p63 (clone PM163AA, pre  
191 diluted, Bio Care), anti-E-cadherin (clone 180223, 1:25 dilution, Invitrogen), anti-HER2  
192 (clone 790 4493, pre diluted, Roche) and anti-CD8 (clone SP16, 1:100 dilution, Spring  
193 Bioscience). For PD-L1, staining was performed using a laboratory developed-test  
194 calibrated with either clone 28-8, (1:100 dilution, Abcam) or clone QR1 (1:100, Quartett,  
195 Germany) and calibrated in comparison to the 22C3 PD-L1 assay. Slides were  
196 deparaffinized in xylene and hydrated in graded alcohol and immunostaining were  
197 performed using BenchMark® XT and BenchMark® Ultra autostainers (Ventana,  
198 Tucson, AZ) and detection was performed using UltraView DAB™ kits (Ventana®  
199 Medical System, Tucson, USA) with amplification for some antibodies. 3,3'-  
200 DiAminoBenzidine (DAB) was used as a chromogen and slides were counterstained  
201 with hematoxylin. P63, GATA3, CK5/6, CK20 and E-cadherin immunostainings were

202 considered as positive when at least 5% of tumor cells were stained at any intensity.  
203 HER2 expression was scored as 0, 1+, 2+ and 3+ according to the ASCO/CAP 2018  
204 HER2 test guideline [9]. HER2 IHC 3+ was considered to be HER2-positive, IHC 2+  
205 HER2-equivocal, and IHC 0 and 1+ HER2-negative. [9] PD-L1 was evaluated in tumor  
206 cells (TC) and immune cells (IC) on the whole infiltrative tumor area. PD-L1 expression  
207 on TC was assessed as the percentage of TC with membranous staining at any  
208 intensity. PD-L1 expression on IC was determined as the proportion of tumor area  
209 occupied by PD-L1-positive IC of any intensity. Within a case with mixed variants, only  
210 the expression of a given marker in the plasmacytoid component was reported in the  
211 plasmacytoid cohort.

### 212 *2.3. Fluorescent In Situ Hybridization*

213 *HER2* amplification was examined by dual-color ZytoLight SPEC ERBB2/D17S122  
214 (ZytoVision, Bremerhaven, Germany) in cases that showed an immunohistochemical  
215 score of 2+ or 3+. All specimens were processed (e.g. demasked, denatured,  
216 hybridized, etc.) according to the manufacturer's instructions. Finally, the slides were  
217 mounted and counterstained with 4',6-diamidino-2'-phenylindole (DAPI) Vectashield  
218 (Vector Laboratories, Burlingame, CA, USA) and analyzed by fluorescence microscopy.  
219 FISH results were analyzed by counting the fluorescent signals in 20 malignant cells.  
220 For each case, the average copy number and the ratio of *HER2* signals to chromosome  
221 17 centromere (CEP17) signals were calculated. *HER2* status by FISH was interpreted  
222 according to the ASCO/CAP 2018 *HER2* test guideline [9].

### 223 *2.4. Image analysis*

224 Image acquisition was performed with a Virtual Slides microscope VS120-SL 2.5  
225 (Olympus, Tokyo, Japan), 20X air objective (0.75 NA). CD8<sup>+</sup> cells were detected using  
226 an algorithm created in Definiens Developer software. Regions of interest (ROI) were  
227 selected manually. The method combined watershed segmentation on DAB staining  
228 and color and morphological characteristics to retrieve automatically CD8<sup>+</sup> cells. It  
229 exports the number of CD8<sup>+</sup> cells and the surface of ROI in  $\mu\text{m}^2$  for each tissue sample.

### 230 *2.5. Statistical analysis*

231 Statistical analysis was performed using Graphpad Prism 8 Software (GraphPad  
232 Software, La Jolla, CA). The overall survivals for patients with plasmacytoid UC at  
233 different stages were compared using the Kaplan-Meier method. Continuous variables  
234 and proportions were compared using Mann-Whitney and Fisher exact tests,  
235 respectively. *P* values less than .05 were considered statistically significant.

## 236 **3. Results**

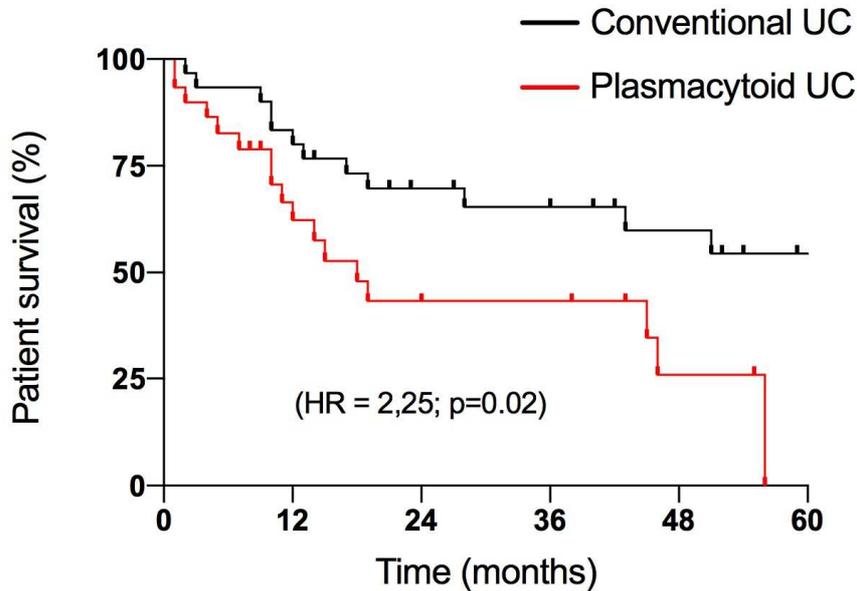
### 237 *3.1. Clinical and demographic findings*

238 The clinicopathological features of plasmacytoid UC cohort and matched controls are  
239 summarized in Table 1. The male to female ratio was 3:1. The mean age was 66 (44-  
240 91) at the time of plasmacytoid UC diagnosis. In the plasmacytoid group, 18 patients  
241 (56%) had locally advanced pathological stage pT3 (n=11; 34%) and pT4 (n=7; 22%).  
242 Tumor samples were provided from diagnostic TURs (n=21) and radical cystectomy  
243 specimen (n=11). Overall, 15 patients (47%) had local-regional lymph node metastases  
244 and 3 patients (9%) had a metastatic disease. The tumors demonstrated a diffusely  
245 infiltrative and solid growth pattern. Regarding the control group, tumor samples were

246 provided from diagnostic TURs (n=19) and radical cystectomy specimen (n=11). The  
 247 median follow-up was 19.6 months (range, 1-56 months) and 32 months (range, 2-134  
 248 months) for the plasmacytoid and the control groups, respectively. The 5-year overall  
 249 survival rate of patients with plasmacytoid UC was significantly lower compared to the  
 250 control group (HR = 2,246; 95% CI=1,069 to 4,716; p=0.02) (Figure 1).

Characteristics	Plasmacytoid UC	Conventional UC	P value
	n=32 (100%)	n=30 (100%)	
Age, years (median, range)	66 (44-91)	70 (45-85)	0.2
Gender			
Male	9 (28%)	9 (30%)	>0.99
Female	23 (72%)	21 (70%)	
Smocking status			
Non smoker	6 (19%)	5 (16%)	>0.99
Former smoker	19 (59%)	18 (60%)	
Active smoker	3 (9%)	1 (3%)	
N/A	4 (13%)	6 (20%)	
Pathological stage			
pT1	3 (9%)	3 (10%)	1
pT2	11 (34%)	11 (36%)	1
pT3	11 (34%)	10 (33%)	>0.99
pT4	7 (22%)	6 (20%)	>0.99
N1-3	15 (47%)	12 (40%)	1
M1	3 (9%)	3 (10%)	1
Pathological features			
<i>In situ</i> carcinoma	17 (53%)	18 (60%)	0.8
Lympho-vascular invasion	20 (63%)	20 (66%)	1
Treatment			
Perioperative chemotherapy	17 (53%)	11 (36%)	0.21
First-line chemotherapy	12 <sup>11</sup> (37%)	15 (50%)	0.44

251 Table 1. Demographic and clinico-pathological characteristics of cohorts with plasmacytoid UC  
252 and conventional high grade UC.



253

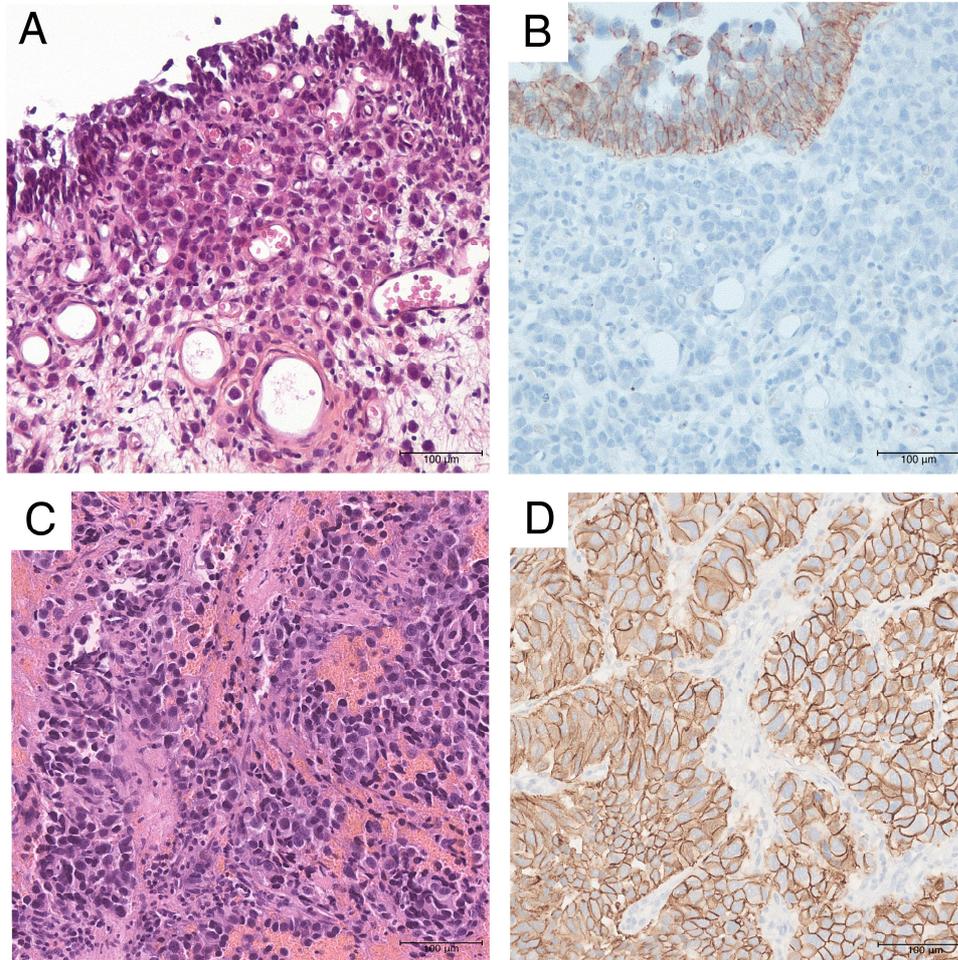
254 Fig. 1. Kaplan–Meier Plots of Overall Survival at 5 years among 32 patients with plasmacytoid  
255 UC (red bar) and 30 patients with conventional high-grade UC (black bar) (HR = 2,246; 95%  
256 CI=1,069 to 4,716; p=0.02). *color should be used for print single column fitting image*

257

### 258 3.3. Morphological features

259 The plasmacytoid component accounted for a mean of 75% (range, 5%-100%) of the  
260 tumor. Plasmacytoid UC consisted in independent cells with eccentrically placed nuclei  
261 and a moderate to abundant eosinophilic cytoplasm arranged in a sheet-like pattern or  
262 showed dyscohesive tumor cells often associated with a dense and fibrous stroma  
263 (Figure 2). Plasmacytoid UC were pure in 11 (34%) cases and mixed with other UC  
264 types in 21 cases (66%), including conventional high grade UC (n=11), neuroendocrine  
265 (n=2), micropapillary (n=6), and squamous cell carcinoma (n=2) variants with 4 cases  
266 showing both micropapillary and conventional high grade UC mixed with the  
267 plasmacytoid component. The plasmacytoid component was predominant in most

268 mixed cases except for 8 cases (6 cases mixed with conventional high grade UC, 1 with  
269 a micropapillary component and 1 case with a squamous cell carcinoma component).



270

271 Fig. 2. Plasmacytoid urothelial carcinoma and urothelial high grade UC. Representative  
272 histopathological features of plasmacytoid UC showing independent cells with eccentrically  
273 placed nuclei and a moderate to abundant eosinophilic cytoplasm (A, hematoxylin  
274 and eosin [H&E], x200). A conventional high grade UC forming clusters of cells with severe  
275 cytologic atypia (C, H&E, x200). Immunohistochemical staining with E-cadherin showing no  
276 expression of E-cadherin in the plasmacytoid tumor and membranous positivity of the  
277 urothelium as an internal positive control (B, E-cadherin, x 200). Immunohistochemical staining  
278 with E-cadherin showing membranous expression in the conventional high grade UC (D, E-  
279 cadherin, x 200). *color should be used for print single column fitting image*

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282 *3.4. Immunohistochemical profiling*

283 *3.4.1. E-cadherin status*

284 Plasmacytoid UC lack E-cadherin expression in 24 cases out of 32 cases (75%). Eight  
285 cases (25%) showed a weak to moderate membranous expression whereas the  
286 majority of controls (80%) showed high E-cadherin expression ( $p < 0.05$ ). Figure 2 shows  
287 loss of nuclear expression of E-cadherin in plasmacytoid UC. Two cases (6%) exhibited  
288 a cytoplasmic dot expression.

289 *3.4.2. Basal/luminal subtype*

290 Table 2 summarizes the expression of basal and luminal markers across plasmacytoid  
291 tumors and controls. Plasmacytoid UC harbored GATA3 and CK20 expression in 31  
292 (97%) and 19 (59%) tumors, respectively (Figure 3). In the control group, GATA3 and  
293 CK20 expression was observed in 26 (86%) and 11 (36%) conventional UC,  
294 respectively. No significant difference was found between both groups regarding the  
295 luminal markers. CK5/6 and p63 expression was observed in 7 (22%) and 13 (40%)  
296 plasmacytoid UC, respectively. In the control group, CK5/6 and p63 were expressed in  
297 17 (56%) and 24 (80%), respectively (Figure 3). We found a significant difference  
298 between both groups regarding the basal markers ( $p < 0.05$ ). Of note, a mixed  
299 expression of luminal and basal markers was observed in 11 (34%) plasmacytoid UC  
300 and 18 (60%) controls.

301

302

IHC Markers		Plasmacytoid UC	Controls	P_value
		No. (%)	No. (%)	
Basal subtype	CK5/6	7 (22%)	17 (56%)	<0.05
	p63	13 (40%)	24 (80%)	<0.05
Luminal subtype	GATA3	31 (97%)	26 (86%)	0.18
	CK20	19 (59%)	11 (36%)	0.08
E-cadherin		8 (25%)	24 (80%)	<0.05

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304 Table 2. Immunohistochemical profile of plasmacytoid UC and conventional high grade UC.

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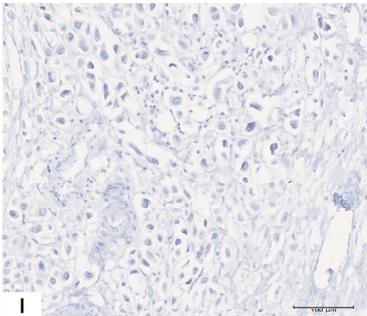
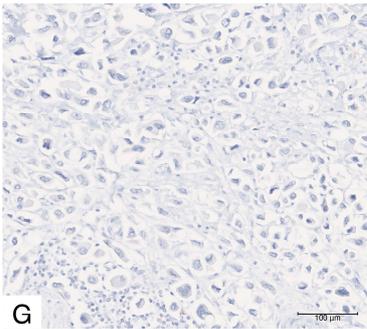
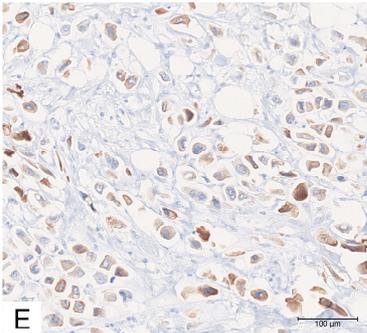
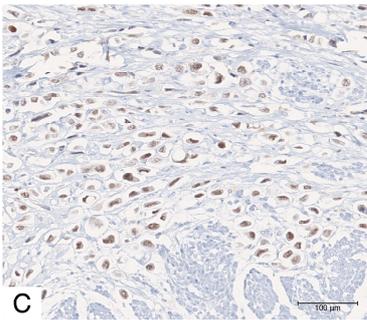
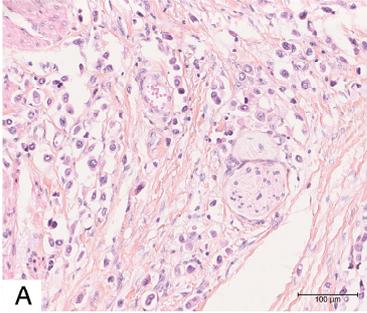
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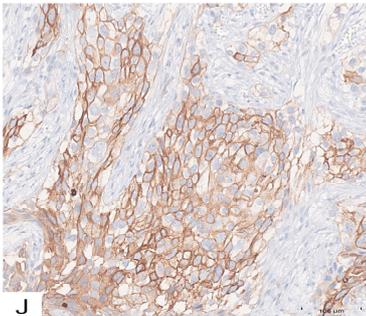
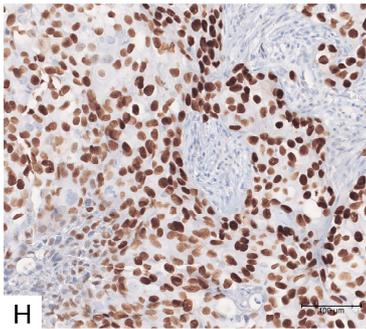
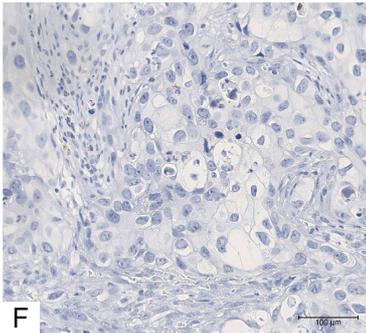
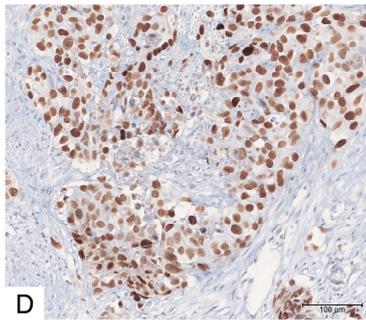
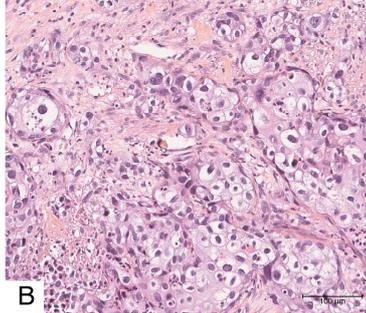
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316

Plasmacytoid UC



High grade conventional UC



318 Fig. 3. Immunohistochemical findings. A plasmacytoid UC (A, H&E ×200) and a high grade  
 319 conventional UC (B, H&E, x 200). A plasmacytoid UC with positive expression of GATA3 (C,  
 320 x200), CK20 (E, x 200) and no expression of p63 (G, x200) and CK5/6 (I, x 200). A high grade  
 321 conventional UC with positive expression of GATA3 (D, x 200), p63 (H, x 200), CK5/6 (J, x 200)  
 322 and no expression of CK20 (F, x 200). *color should be used for print, 1.5-column fitting image*

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### 324 3.4.3. *HER2* expression and amplification

325 A membranous expression of *HER2* protein was present in 9 (28%) plasmacytoid UC  
 326 with 1 case (3%) scored 1+, 6 cases (19%) scored 2+ and 2 cases (6%) scored 3+  
 327 (Table 3). The number of cases with *HER2*+ expression scored 2+ and 3+ was  
 328 significantly higher in plasmacytoid UC than in controls (p<0.01). In the control group, 5  
 329 cases (17%) showed score 1+ *HER2* expression and 25 cases (83%) did not exhibit  
 330 *HER2* expression. *HER2* amplification was analyzed by FISH on 8 out 9 cases showing  
 331 an expression scored 2+ or 3+. Only 1 case showed *HER2* amplification with a number  
 332 of *HER2* copies >10. Representative images of *HER2* detection are shown in Figure 4.

<b>HER2 expression</b>	<b>Plasmacytoid UCs</b>	<b>Controls</b>
	<b>No (%)</b>	<b>No (%)</b>
- score 0	23 (72%)	25 (83%)
- score 1+	1 (3%)	5 (17%)
- score 2+	6 (19%)	0 (0%)
- score 3+	2 (6%)	0 (0%)

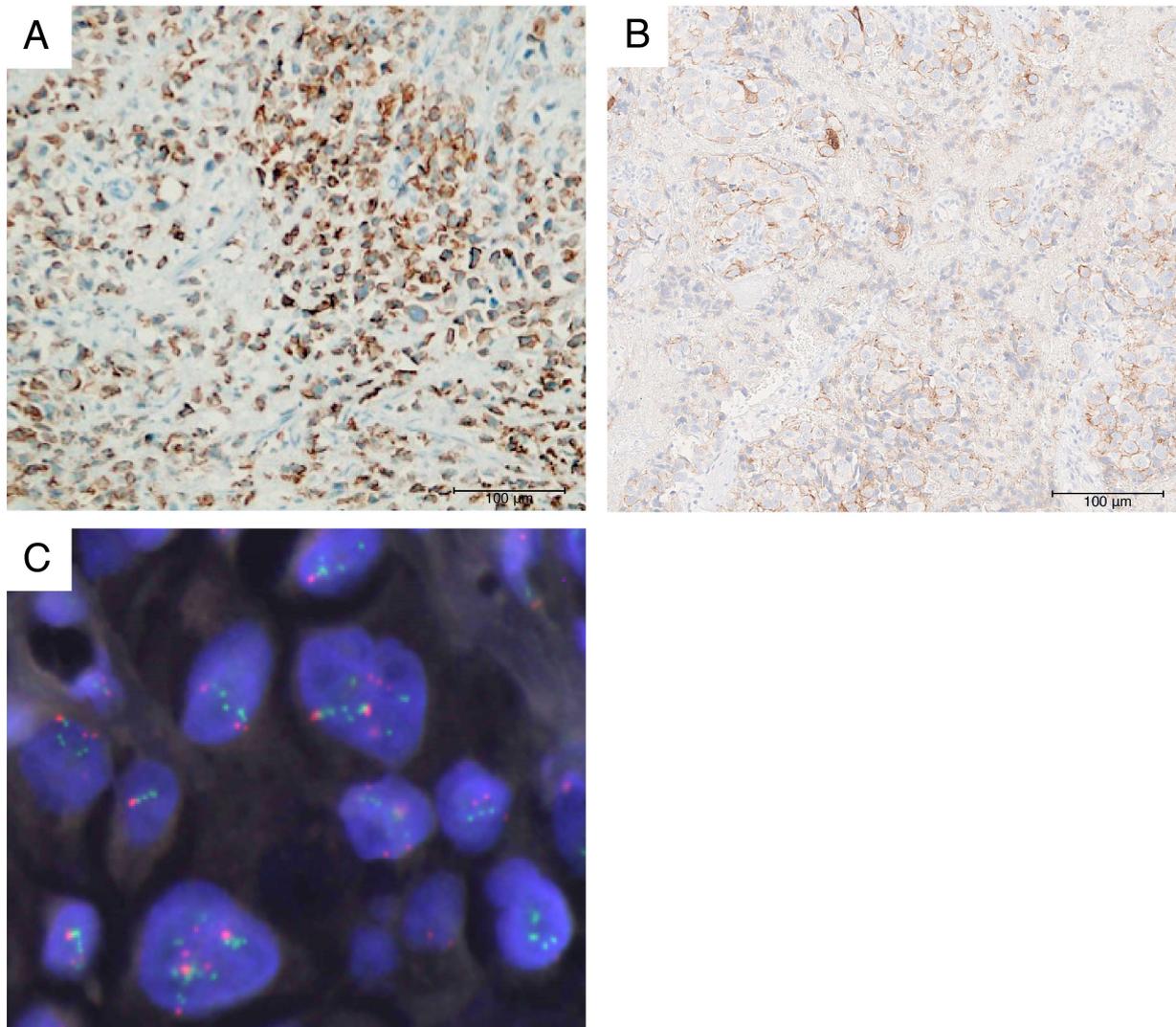
338 Table 3. *HER2* expression and *HER2* amplification in plasmacytoid UC and conventional high  
 339 grade UC.

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344

345 Fig. 4. HER2 expression and Fluorescent In Situ Hybridization findings. A plasmacytoid UC  
 346 showing a diffuse score 3+ HER2 IHC positivity (A, HER2 x100) and *HER2* amplification  
 347 (HER2/CEP17 ratio=3.6) by FISH (C). A conventional high grade UC showing a score 1+ HER2  
 348 positivity (B, HER2 x100). *color should be used for print, 1.5-column fitting image*

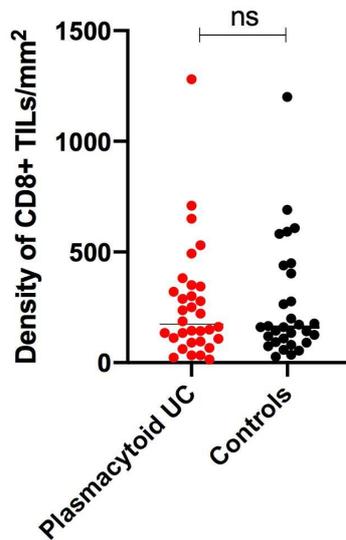
349

#### 350 3.4.4. Tumor Immune microenvironment profile

351 The mean density of CD8<sup>+</sup> cells infiltrating the tumor (number of CD8<sup>+</sup> TILs/mm<sup>2</sup>) was  
 352 259.1 CD8<sup>+</sup> cells/mm<sup>2</sup> (median=162.0 CD8<sup>+</sup> cells/mm<sup>2</sup>) in the plasmacytoid UC group.  
 353 Similarly, we found a mean density of 261.0 CD8<sup>+</sup> cells/mm<sup>2</sup> (median 160.5 CD8<sup>+</sup>

354 cells/mm<sup>2</sup>) in the control group (Figure 5). No significant difference was observed  
355 between both groups (p=0.9). PD-L1 was expressed on tumor cells (TC) in 1  
356 plasmacytoid UC (3%) and 9 controls (30%). PD-L1 expression on TC was significantly  
357 lower in plasmacytoid UC compared to controls (p<0.05). By contrast, PD-L1 expression  
358 on immune cells (IC) was not statistically different between both cohorts (p=0.3) (Table  
359 4). Figure 6 depicts CD8 expression in T cells and PD-L1 expression on TC and IC in  
360 both conventional UC and plasmacytoid UC.

361



	<b>Plasmacytoid UC</b>	<b>Controls</b>	<b>P value</b>
<b>Tumor cells</b>			
- 0 %	29	21	
- 1 - ≤5 %	0	8	<0.05
- >5 %	1	1	
<b>Immune cells</b>			
- 0 %	23	17	
- 1 - ≤5 %	8	10	0.3
- >5 %	1	3	

372

373 Table 4. PD-L1 expression in Tumors Cells (TC) and Immune Cells (IC) in plasmacytoid UC and  
374 conventional high grade UC.

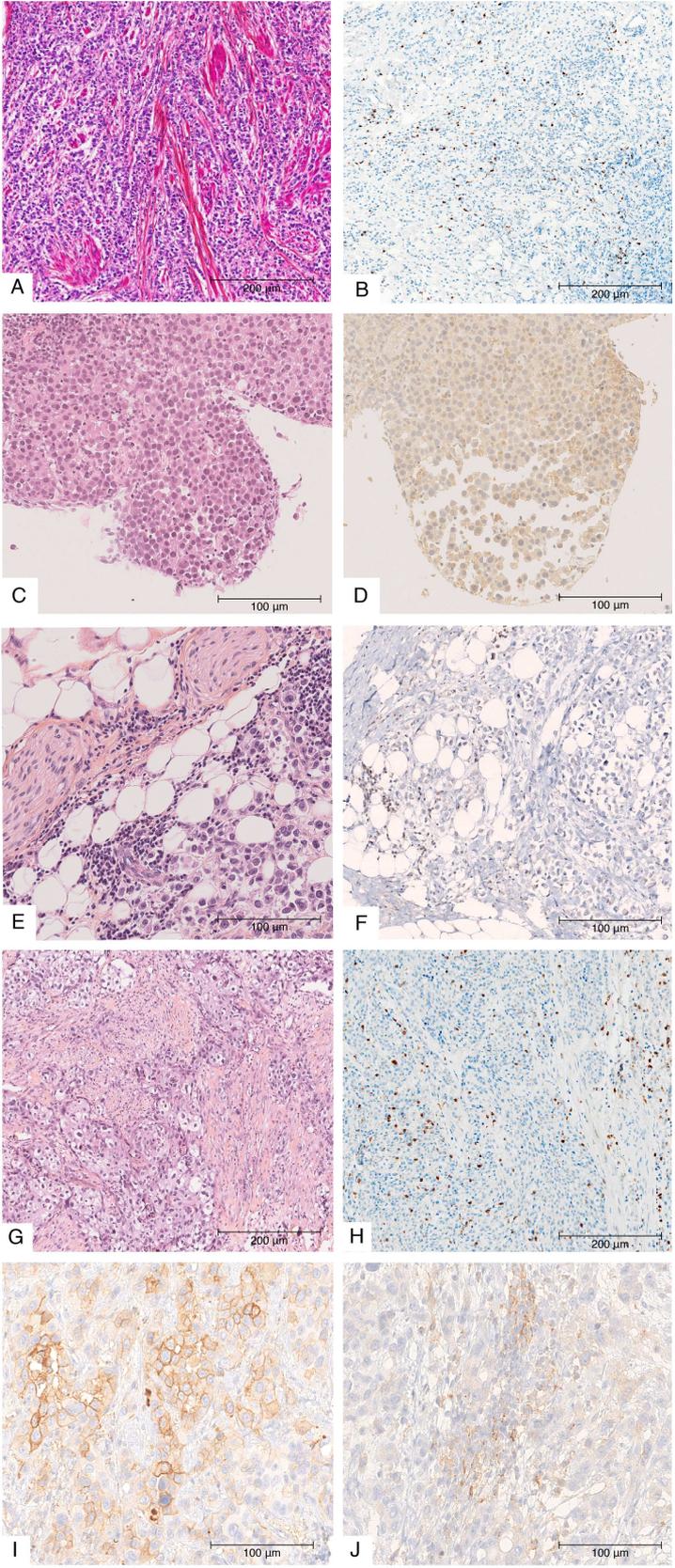
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381 Fig. 6. CD8 and PD-L1 expression in plasmacytoid UC and conventional high grade UC. H&E  
382 images of plasmacytoid UC (A, ×100, C and E ×200) and a high grade conventional UC (E,  
383 ×200 and G, x 100). CD8 expression in T cells in a plasmacytoid UC (B, x100), and in a  
384 conventional UC (H, x 100). PD-L1 expression in TC in a plasmacytoid UC (D, x200) and in a  
385 conventional UC (I, x 200). PD-L1 expression in IC in a plasmacytoid UC (F, x200) and in a  
386 conventional UC (J, x 200). *color should be used for print, 1.5-column fitting image*

387

#### 388 **4. Discussion**

389 In this study, we characterized the histopathological subtype and the tumor immune  
390 microenvironment profile of plasmacytoid variant of UC. We compared a cohort of  
391 plasmacytoid UC (n=32) and matched conventional UC cases (n=30) on the basis of  
392 clinicopathological parameters. We found that plasmacytoid UC were characterized by a  
393 luminal profile, and a complete loss of E-cadherin expression in 75% of the tumors.  
394 Additionally, the density of tumor-infiltrating CD8<sup>+</sup> T-cells and the percentage of PD-L1  
395 expression on immune cells were similar to controls with conventional UC. Our study  
396 identifies new biological insights of plasmacytoid UC and suggests potential clinical  
397 implications for immunotherapeutic strategies in this aggressive disease.

398 These findings are important at different levels. First, several groups have identified  
399 muscle-invasive bladder cancer (MIBC) molecular subtypes based on gene expression  
400 profile [10–17]. Molecular classifications of bladder cancer based on transcriptomic  
401 profile have highlighted two main subtypes, which include the luminal and the basal  
402 subtypes. Phenotypically, basal tumors express CK5/6 and FOXA1 and luminal tumors  
403 express luminal markers such as GATA3 and cytokeratin CK20 [1,2]. The identification  
404 of such markers eventually led to the development of an immunohistochemical  
405 signature that segregates molecular subtypes with over 90% accuracy [21]. Based on

406 such markers, we found that plasmacytoid UC tend to belong to the luminal subgroup  
407 with a high expression of luminal markers GATA3 and CK20 together with a low  
408 expression of basal markers CK5/6 and p63. This finding is consistent with prior studies,  
409 which showed a high expression of GATA3 and CK20 in plasmacytoid UC [22–25].  
410 Additionally, our study showed a significantly lower expression of basal markers as  
411 compared to conventional UC, which reinforces the internal coherence of the luminal  
412 profile of such tumors. Recent molecular studies have revealed molecular heterogeneity  
413 among the luminal subtypes with potential clinical utility for predicting outcomes and  
414 response to treatment [26]. Whether luminal-type bladder tumors may be more sensitive  
415 or resistant to immunotherapy remain controversial [8,27,28].

416 The luminal subtype has been recently segregated into three different subgroups, which  
417 include the luminal papillary, luminal non-specified, luminal unstable subtypes. Similarly  
418 to our findings, bladder cancers with micropapillary and nested variant histologies have  
419 been classified in the luminal subtype with aggressive clinical behavior [29–31]. Indeed,  
420 bladder cancer with luminal molecular subtype and variant histologies are poor  
421 responder to neoadjuvant chemotherapy [31,32]. Future research should focus on  
422 discriminating aggressive variants among the luminal subtype to identify which  
423 treatment is best for which subgroup [26]. We investigated HER2 protein expression  
424 and *HER2* amplification in our cohort since HER2 alterations were higher in the luminal  
425 groups compared to the basal groups in the TCGA cohort [33]. Micropapillary UC  
426 variant which tends to belong to the luminal subtype showed a high frequency of HER2  
427 alterations [34]. We showed that HER2 expression and amplification were higher in  
428 plasmacytoid UC compared to controls, further supporting their affiliation to the luminal

429 subtype. Altogether, HER2 status could be also considered as a potential targeted  
430 therapeutic strategy in some plasmacytoid UC cases.

431 Second, our study demonstrates that plasmacytoid UC had similar PD-L1 expression on  
432 immune cells as compared to controls. Indeed, PD-L1 may co-opt checkpoint inhibitory  
433 receptors to suppress effector T cell function [35]. Similarly to our findings, Reis et al.  
434 reported that a proportion of plasmacytoid UC expressed PD-L1 protein, as assessed  
435 by different antibodies, at different cutoff levels and methods of evaluation (tumor cells  
436 and/or immune cells) [36]. Paradoxically, conventional high grade UC showed a  
437 significantly higher number of PD-L1 tumor cells compared to plasmacytoid UC which  
438 could be explained by the fact that distinct mechanisms regulate PD-L1 expression on  
439 immune cells and tumor cells [37]. Although the role of PD-L1 as a biomarker of  
440 response to immune checkpoint blockade in UC remains controversial, this finding  
441 suggests that anti-PD-1 or anti-PD-L1 monoclonal antibodies may be effective on  
442 plasmacytoid UC. In addition, we found that the density of CD8<sup>+</sup> T-cells was similar  
443 between plasmacytoid and conventional UC. A significant enrichment of stromal TILs in  
444 T1 bladder tumors with variant histology ( $p = 0.01$ ) has been identified in one of our  
445 previous studies [38]. Overall, these data suggest that plasmacytoid UC are associated  
446 with an increased adaptive immune response.

447 Besides these immune parameters, tumor mutation burden (TMB) has been recently  
448 proposed as a biomarker of response to immune checkpoint blockade (ICB), including  
449 plasmacytoid UC [3,36,39,40]. Together, these data suggest that plasmacytoid UC are  
450 solid tumors with similar T cell infiltration as compared to conventional UC, which  
451 suggest potential response to ICB. While the presence of a variant differentiation in

452 invasive UC is an exclusion criterion for most clinical trials testing ICB in invasive UC,  
453 our results provide rationale to question the place of such therapeutic strategy in the  
454 management of plasmacytoid UC. A recent study evaluated atezolizumab in locally  
455 advanced/metastatic UC with renal insufficiency or UC with mixed variant histology and  
456 showed a clinical benefit [41].

457 Third, our study revealed that a complete loss of E-cadherin expression occurred in  
458 75% of plasmacytoid UC. Similarly, Al Ahmadie et al. identified a specific non sense  
459 mutation of *CDH1* (*Cadherin 1*) gene in plasmacytoid UC, leading to E-cadherin protein  
460 expression loss, and an increased migratory capability [3]. This biological characteristic  
461 reinforces the crucial need to perform radical surgery under the strict principle of en bloc  
462 resection in order to secure negative perivesical soft tissue margin, and perivesical soft  
463 tissue surfaces to avoid local spreading of cancer cells [42,43].

464 This study has some limitations, which are inherent to its retrospective nature. The IHC  
465 panel used to define molecular subtype is not exhaustive and could be considered as a  
466 limiting parameter. Bladder cancer is often histologically and molecularly heterogeneous  
467 within a given patient, supporting that subtyping bladder cancer is complex and can be  
468 restricting [44]. The heterogeneity of basal and luminal marker expressions within  
469 conventional UC in our study illustrated this complexity.

## 470 **5. Conclusion**

471 Overall, our data showed that plasmacytoid UC tend to belong to the luminal molecular  
472 subgroup with aggressive clinical behavior. In our series, plasmacytoid UC harbored  
473 similar CD8<sup>+</sup> T cells density and PD-L1 expression on immune cells as compared to

474 conventional UC. Together, these data suggest that patients with plasmacytoid UC may  
475 benefit from immune checkpoint blockade similarly to conventional UC. This strategy is  
476 currently investigated in in the ABACUS-02 trial (NCT04624399) in a neoadjuvant setting  
477 prior to radical cystectomy.

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