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ARTICLE

Treatment algorithm and prognostic factors for patients with stage I–III carcinoma of the anal canal: a 20-year multicenter study

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Abstract

Despite a growing incidence in developed countries and a recent improved understanding of its pathogenesis, anal cancer management has not evolved over the past decades and drug combination used as first-line regimen still largely depends on clinician preferences. Aiming at paving the way for precision medicine, a large cohort of 372 HIV-negative patients diagnosed over a 20-year time period with locally advanced anal carcinoma was collected and carefully characterized at the clinical, demographic, histopathologic, immunologic, and virologic levels. Both the prognostic relevance of each clinicopathological parameter and the efficacy of different concurrent chemoradiation strategies were determined. Overall, the incidence of anal cancer peaked during the sixth decade (mean: 63.4) and females outnumbered males (ratio: 2.51). After completion of treatment, 95 (25.5%) patients experienced progression of persistent disease or local/distant recurrence and 102 (27.4%) died during the follow-up period (median: 53.8 months). Importantly, uni-multivariate analyses indicated that both negative HPV/p16^{ink4a} status and aberrant p53 expression were far better predictors for reduced progression-free survival than traditional risk factors such as tumor size and nodal status. As for overall survival, the significant influences of age at diagnosis, p16^{ink4a} status, cTNM classification as well as both CD3^{+} and CD4^{+} T-cell infiltrations within tumor microenvironment were highlighted. Cisplatin-based chemoradiotherapy was superior to both radiotherapy alone and other concurrent chemoradiation therapies in the treatment of HPV-positive tumors. Regarding their HPV-uninfected counterparts, frequent relapses were observed, whatever the treatment regimen administered. Taken together, our findings reveal that current anal cancer management and treatment have reached their limits. A dualistic classification according to HPV/p53 status should be considered with implications for therapy personalization and optimization.

Introduction

With an estimated 48,500 new cases diagnosed worldwide in 2018 [1], the incidence of anal cancer has substantially raised since the early 80's [2]. Although less common than adenocarcinoma of the colon or rectum, both intraepithelial and invasive neoplasms of the anal canal are no longer considered as rare by most clinicians, pathologists, and epidemiologists. From a pathogenesis standpoint, anal cancer is undoubtedly more similar to lower gynecological tract tumors than it is to other gastrointestinal malignancies. Indeed, most (~85%) anal (pre)neoplastic lesions display a squamous differentiation [3], are caused by a carcinogenic human papillomavirus (HPV) infection and are related to

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risk factors such as a high lifetime number of sexual partners or an immunocompromised status (e.g., HIV positivity) [4, 5].

Although surgery (especially to remove early-stage tumors such as superficially invasive lesions) or radiotherapy alone (with fragile patients or older than 80) are sometimes advisable, the vast majority of tumors arising from the anal canal are treated with a combination of chemotherapy and radiation. Concurrent 5-fluorouracil (5-FU)/mitomycin plus radiotherapy (45–59.4 Gy) has been constituting the preferred primary treatment option for patients with locally advanced anal carcinoma for several decades [6, 7]. Most patients who have experienced treatment failure undergo surgical excision (usually a salvage abdominoperineal resection), affecting their quality of life. Given that locoregional relapse is well-known to occur in ~35% of patients, some treatment alternatives using cisplatin or capecitabine (xeloda®) were tested [8–11]. Of note, several clinical trials evaluating immune checkpoint inhibitors (anti-PD-1, PD-L1, and/or CTLA-4) in patients with refractory or metastatic anal squamous cell carcinoma (SCC) are still ongoing (e.g., NCT02314169, NCT02919969) [12]. Despite some promising results (especially with cisplatin-based therapy) [11], conflicting results have been reported [9, 10]. The limited number of enrolled patients and/or the (too) wide eligibility criteria (e.g., absence of reliable tumor characterization, unknown HIV status) are very likely to explain these existing discrepancies. Indeed, potential risk factors such as HPV status and disruptive *TP53* mutations as well as the existence of two distinct subtypes of anal SCC with different cellular origin (squamous zone vs. transitional zone) were recently highlighted [13–15]. Moreover, the predictive value of some T-cell subsets (e.g., $CD8^+$ and $PD\text{-}1^+$) infiltrating tumor stroma was revealed in the context of other HPV-driven cancers (both head and neck and cervical SCC) [16–19]. Therefore, in an era of precision medicine, it is reasonable to think that not all anal cancer patients should be managed/treated in the same way.

The aim of our large retrospective study was (1) to identify HIV-negative non-metastatic anal cancer patients at high risk of recurrence/progression, (2) to define robust prognostic markers, (3) to determine the efficacy of different chemoradiotherapy regimens and, ultimately, (4) to pave the way for a future personalization of treatment algorithms.

Material and methods

Patient selection and clinical data retrieval

A total of 372 patients treated for primary stages I to III (according to the American Joint Committee on Cancer, 8th edition) anal SCC between January 1998 and December 2017 were selected. The consultations and treatments were taking place in five different University/Regional Medical Centers located in Belgium [Liege ($n = 83$)] or north-eastern France [Nancy ($n = 71$), Dijon ($n = 101$), Strasbourg ($n = 72$), Besançon ($n = 45$)]. Paraffin-embedded tissue specimens (biopsies or surgical resections) from each patient were retrieved from pathology archives and processed/archived in the Biobanks of the University Hospital of Liege or University Hospital of Besançon (BB-0033–00024) throughout the project. Both original diagnoses and tumor differentiation were confirmed by experienced pathologists (SV-D and PD). In order to avoid bias which could affect the analysis, patients with incomplete clinicopathological information, metastatic (stage IV) disease, or treated for a glandular neoplasm (anal adenocarcinoma or rectal tumor spreading downward within the anal canal) were excluded. Patients who directly received non-curative-intent treatments (palliative care), who were immunosuppressed (HIV-positive or transplant recipients) as well as patients diagnosed with a cancerous lesion entirely detected in the anal margin (defined by the presence of hair follicles and sweat glands) were not taken into consideration either. Clinicopathological information [gender, age at diagnosis, tumor size, nodal and HIV statuses, treatment details (surgical procedures, radiotherapy doses, chemotherapeutic agents) and follow-up data] was collected for all patients from personal health records. This study (data collection and experimental protocols) was approved by the institutional review board of the respective institutions (Belgium: #2020/51; France: #F1962-CAPINDEPTH).

Immunohistochemistry

After deparaffinization and rehydration in graded alcohols, the activity of endogenous peroxidases was blocked using 3% H_2O_2 in methanol for 5 min. Antigens were then retrieved in 10 mM citrate buffer (pH 6) (Sigma-Aldrich, St Louis, MO, USA) or in 10 mM Tris/1 mM EDTA solution (pH 9) (Invitrogen, Carlsbad, CA, USA) for 11 min at 120 °C in a pressure cooker. Before incubating the tissue sections with the primary antibodies for 1 h at room temperature, the non-specific binding sites were blocked using serum-free Protein Block reagent (Dako/Agilent Technologies, Glostrup, Denmark). The following antibodies were used for the primary reaction: anti-$p16^{ink4a}$ (clone E6H4; CINtec Histology-Roche, Basel, Switzerland), anti-p53 (clone DO-7; Ventana, Medical Systems, Tucson, AZ, USA), anti-cytokeratin 7 (CK7) (clone SP52; Ventana Medical Systems,), anti-Ki67 (clone 30–9; Ventana Medical Systems), anti-PD-1 (clone NAT105; Abcam, Cambridge, UK), anti-CD3 (clone 2GV6; Ventana Medical Systems), anti-CD4 (clone SP35; Ventana Medical Systems), anti-CD8 (clone SP57; Ventana Medical

Systems) and anti-carbonic anhydrase 9 (CA IX) (ab15086, Abcam). The secondary reaction (immunoperoxidase staining) was performed using the mouse/rabbit EnVision detection system (Dako) and positive cells were finally visualized using SignalStain DAB Substrate Kit (Cell Signaling, Danvers, MA, USA). Mouse and rabbit control IgGs (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as negative controls.

Immunostaining assessment

All immunolabelled tissues were evaluated by independent investigators (senior pathologists) and the entire cancerous lesion was considered. As previously described [15, 20], $p16^{ink4a}$ and CK7 immunostainings were scored as positive when a diffuse immunoreactivity was detected in the large majority (>75%) of tumor cells. The detection of 1–49% cancer cells displaying a nuclear p53 expression was considered as non-aberrant. In contrast, p53 staining was classified as aberrant when 0% or ≥50% positive cells with moderate or strong intensity were observed [21]. The proliferation index (percentage of Ki67-positive cells) of a given tumor was stratified as follows: 0–25%, 26–50%, 51–75%, and >75%. Both the intensity (negative, low, moderate, or strong) and the extent (undetectable, 0–25%, 26–50%, 51–75%, and >75%) of CA IX immunostained tissues were assessed, according to an arbitrary scale. Regarding T-cell subpopulations ($CD3^+$, $CD4^+$, $CD8^+$, and $PD\text{-}1^+$) infiltrating tumor microenvironment, the number of positive cells per mm^2 was determined by computerized counts (QuPath 0.2.0 software for digital pathology image analysis) and verified by manual counting [22].

HPV genotyping

Following DNA extraction (QIAamp DNA FFPE Tissue Kit; Qiagen, Hilden, Germany), HPV genotyping of French samples was performed using the INNO-LiPA HPV Genotyping Extra test (Innogenetics, Gent, Belgium). The Abbott RealTime High-Risk HPV assay (Abbott, Wiesbaden, Germany) was used for characterizing HPV infections of tissue specimens collected in Belgium. Both assays allow the detection of all WHO/IARC-classified carcinogenic HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and show equal performances (high sensitivity) with formalin-fixed tissues [23].

In situ hybridization

HPV infection was visualized and further confirmed on tissue sections by in situ hybridization. The INFORM HPV III Family 16 Probe cocktail (allowing the detection of HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) was used according to supplier's recommendations (Ventana Medical Systems).

Statistical analysis

GraphPad Prism 5 and R statistical software packages were used to conduct the statistical analyses. Differences were considered statistically significant when $p < 0.05$. Depending on the number of variables (two or more), the comparison of clinicopathological data between independent groups was performed using a Fisher's exact test or a χ^2 test. The Kaplan-Meier method was used to estimate both the overall (OS) and progression-free (PFS) survivals. The OS was calculated from the date of original diagnosis/biopsy to the date of death (from any cause). The events included in PFS were the progression of persistent diseases, recurrences at the local/primary site, and distant metastases. When a patient was still alive without event occurrence at last follow-up visit, this latter date was used as the end point. The survival distribution between separate groups was compared using a Log-rank (Mantel-Cox) test. The prognostic value [hazard ratio (HR) with 95% confidence interval (95% CI)] of each clinicopathological variable was determined in a univariate analysis. Potential risk factors with $p < 0.25$ in univariate analysis were incorporated in a subsequent multivariate analysis based on the Cox regression model. The analysis was then refined using a stepwise method with $p < 0.1$ for removal. The data were compared as follows: age: <60 vs. ≥60; gender: men vs. women; $HPV/p16^{ink4a}$ status: positive vs. negative; HPV infection: single vs. multiple; tumor origin: non-keratinazing squamous zone vs. transitional zone; proliferative index: <50% vs. >50%; p53 staining: non-aberrant vs. aberrant; tumor differentiation: well/moderate vs. poor/basaloid; $CD3^+$ cells: quarter (Q) 2/Q3/Q4 vs. Q1; $CD4^+$ cells: quarter Q2/Q3 vs. Q1/Q4; $CD8^+$ cells: >median vs. <median; $PD\text{-}1^+$ cells: Q2/Q3/Q4 vs. Q1; cT: T1/T2 vs. T3/T4; cN: N + vs. N-; Tumor stage: I-IIb vs. IIIa-IIIc.

Results

Study population: demographic and clinical features

After applying strict inclusion and exclusion criteria (i.e., HIV-negative, non-metastatic squamous diseases with both available tumor tissues and extensive clinicopathological data), 372 patients with stages I to IIIc anal carcinoma were selected. This cohort included four (4/372, 1.1%) superficially invasive SCC and one (1/372, 0.3%) verrucous carcinoma. The mean age was 63.4 years (ranged from 31 to 98) and females outnumbered males (female/male ratio: 2.51). Approximately 60% (225/368, 61.1%) of study

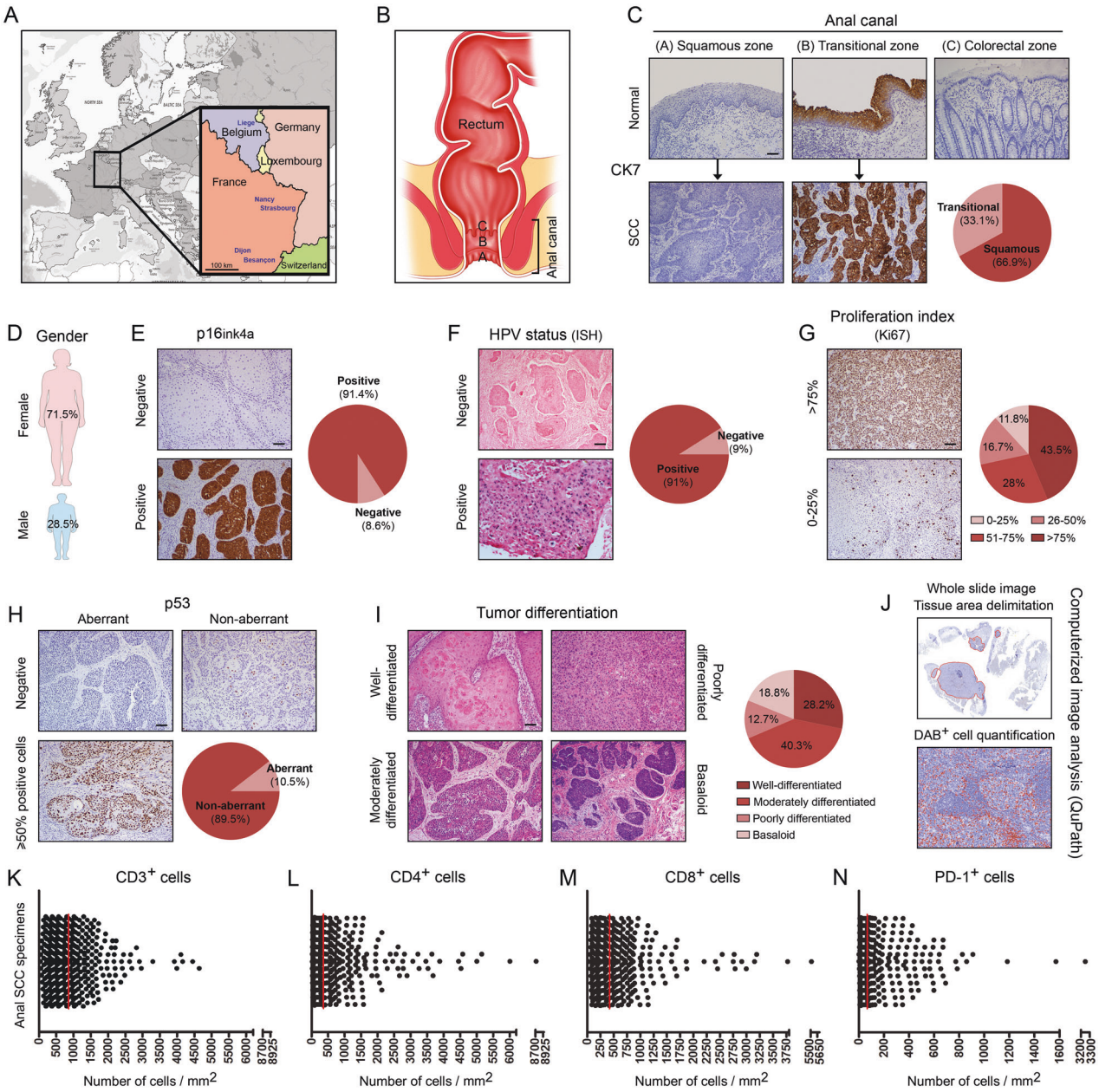

Fig. 1 Description, illustration, measurement of assessed clinicopathological variables, and general characteristics of the study population ($n = 372$). **a** University/regional hospitals participating in the present multicenter study. **b** Schematic representation of the lower gastrointestinal tract. **c** Histology of the different epithelial tissues (squamous, transitional "urothelium-like" and colorectal) lining the anal canal. The specific anti-CK7 immunoreactivity displayed by both the normal urothelium-like epithelium and neoplastic lesions arising from the transitional zone should be noticed. Based on CK7 expression, two region-specific subtypes of anal SCC (squamous vs. transitional) can be easily and precisely distinguished. **d** Percentage of anal canal cancers by gender. **e** $p16^{ink4a}$ expression, **f** HPV status, **g** proliferative index (percentage of Ki67-positive tumor cells), **h** p53 immunoreactivity, and **i** degree of differentiation exhibited by anal canal SCC. Note the diffuse $p16^{ink4a}$ immunoreactivity displayed by the large majority of neoplasms, in keeping with the carcinogenic HPV infection detected by both genotyping assay and in situ hybridization (ISH). **j** Illustration of the different steps followed for computerized DAB-positive immune cell quantification (QuPath). **k** $CD3^+$, **l** $CD4^+$, **m** $CD8^+$, and **n** $PD\text{-}1^+$ T-cell infiltration within tumor microenvironment was determined for each tissue specimen and the number of cells was reported to tumor area (mm^2). The median (in red) is shown. The scale bar represents 100 µm.

patients had no sign of spread to lymph nodes at diagnosis and patients were distributed among each discrete cancer staging category as follows: I: 17.8%; IIa: 33.1%; IIb: 5.5%; IIIa: 20.8%; IIIb: 4.4%; IIIc: 18.4%. Due to the lack of precise information regarding tumor size or nodal status, tumor stage was undetermined in 7 out of 372 (1.9%) patients. About two thirds (247/372, 66.4%) of patients were treated with combined chemoradiotherapy while most

Table 1 Demographic and patient characteristics according to disease recurrence/progression.

Characteristics	No recurrence/progression ($n = 277$) (74.46%)	Treatment failure ($n = 95$) (25.54%)	P value
Age at diagnosis (31–98 years)			$p = 0.718$
<60	115 (41.52%)	42 (44.21%)	
≥60	162 (58.48%)	53 (55.79%)	
Gender			$p = 0.236$
Male	74 (26.71%)	32 (33.68%)	
Female	203 (73.29%)	63 (66.32%)	
HPV status			$\mathbf{p < 0.0001}$
Negative	13 (4.69%)	19 (20%)	
Positive	252 (90.98%)	71 (74.74%)	
Undetermined	12 (4.33%)	5 (5.26%)	
HPV infection			$p = 1.00$
Single	234 (234/252, 92.86%)	66 (66/71, 92.96%)	
Multiple	18 (18/252, 7.14%)	5 (5/71, 7.04%)	
HPV genotypes			$p = 0.699$
HPV16	233 (233/252, 92.46%)	68 (68/71, 95.77%)	
HPV18	7 (7/252, 2.78%)	1 (1/71, 1.41%)	
Others	42 (42/252, 16.67%)	10 (10/71, 14.08%)	
p16ink4a staining			$\mathbf{p < 0.0001}$
Negative	12 (4.33%)	20 (21.05%)	
Positive	265 (95.67%)	75 (78.95%)	
Tumor origin (based on CK7 staining)			$p = 0.801$
Squamous zone	184 (66.43%)	65 (68.42%)	
Transitional zone	93 (33.57%)	30 (31.58%)	
Proliferative index (Ki67)			$p = 0.182$
≤25%	35 (12.64%)	9 (9.47%)	
26–50%	40 (14.44%)	22 (23.16%)	
51–75%	76 (27.43%)	28 (29.47%)	
>75%	126 (45.49%)	36 (37.90%)	
p53 status			$\mathbf{p = 0.011}$
Aberrant (0 or >50%)	22 (7.94%)	17 (17.89%)	
Non aberrant	255 (92.06%)	78 (82.11%)	
Tumor differentiation			$p = 0.597$
Well-differentiated	75 (27.08%)	30 (31.58%)	
Moderately differentiated	110 (39.71%)	40 (42.11%)	
Poorly differentiated	38 (13.72%)	9 (9.47%)	
Basaloid	54 (19.49%)	16 (16.84%)	
$CD3^+$ cells (median: 839.2 cells/mm^2)			$p = 0.895$
First quarter (<448.8 cells/mm^2)	68 (24.55%)	25 (26.31%)	
Second quarter (448.8–839.2 cells/mm^2)	72 (25.99%)	21 (22.11%)	
Third quarter (839.2–1306 cells/mm^2)	69 (24.91%)	24 (25.27%)	
Fourth quarter (>1306 cells/mm^2)	68 (24.55%)	25 (26.31%)	

Table 1 (continued)

Characteristics	No recurrence/progression ($n = 277$) (74.46%)	Treatment failure ($n = 95$) (25.54%)	P value
$CD4^+$ cells (median: 359.2 cells/mm^2)			$p = 0.556$
First quarter (<140.1 cells/mm^2)	71 (25.63%)	22 (23.16%)	
Second quarter (140.1–359.2 cells/mm^2)	68 (24.55%)	25 (26.32%)	
Third quarter (359.2–924.6 cells/mm^2)	73 (26.35%)	20 (21.05%)	
Fourth quarter (>924.6 cells/mm^2)	65 (23.47%)	28 (29.47%)	
$CD8^+$ cells (median: 421.1 cells/mm^2)			$p = 0.792$
First quarter (<235.6 cells/mm^2)	69 (24.91%)	24 (25.26%)	
Second quarter (235.6–421.1 cells/mm^2)	66 (23.83%)	27 (28.42%)	
Third quarter (421.1–739.8 cells/mm^2)	72 (25.99%)	21 (22.11%)	
Fourth quarter (>739.8 cells/mm^2)	69 (24.91%)	23 (24.21%)	
Unknown	1 (0.36%)		
PD-1^+ cells (median: 66.8 cells/mm^2)			$p = 0.966$
First quarter (<30.1 cells/mm^2)	68 (24.55%)	25 (26.32%)	
Second quarter (30.1–66.8 cells/mm^2)	69 (24.91%)	24 (25.26%)	
Third quarter (66.8–164.7 cells/mm^2)	71 (25.63%)	22 (23.16%)	
Fourth quarter (>164.7 cells/mm^2)	69 (24.91%)	24 (25.26%)	
cTNM			
cT			$\boldsymbol{p = 0.047}$
T1–T2	204 (73.65%)	59 (62.11%)	
T3–T4	71 (25.63%)	35 (36.84%)	
Unknown	2 (0.72%)	1 (1.05%)	
cN			$\boldsymbol{p = 0.029}$
N-	177 (63.90%)	48 (50.53%)	
N1	51 (18.41%)	17 (17.89%)	
N2	19 (6.86%)	13 (13.69%)	
N3	27 (9.75%)	16 (16.84%)	
Unknown	3 (1.08%)	1 (1.05%)	
cM			/
M−	277 (100%)	95 (100%)	
M+	0 (0%)	0 (0%)	
Tumor stage (AJCC 8th edition)			$\boldsymbol{p = 0.057}$
Stage I	55 (19.86%)	10 (10.53%)	
Stage IIa	93 (33.57%)	28 (29.47%)	
Stage IIb	16 (5.78%)	4 (4.21%)	

Table 1 (continued)

Characteristics	No recurrence/progression ($n = 277$) (74.46%)	Treatment failure ($n = 95$) (25.54%)	P value
Stage IIIa	56 (20.22%)	20 (21.05%)	
Stage IIIb	11 (3.97%)	5 (5.26%)	
Stage IIIc	41 (14.80%)	26 (27.37%)	
Stage IV	0 (0%)	0 (0%)	
Unknown	5 (1.80%)	2 (2.11%)	
Primary treatment			$p = 0.98$
Chemotherapy	0 (0%)	0 (0%)	
Radiotherapy	86 (31.05%)	29 (30.53%)	
Chemoradiotherapy	183 (66.06%)	64 (67.37%)	
Surgery	8 (2.89%)	2 (2.10%)	
Geographical location			$p = 0.585$
Besançon (France)	35 (12.64%)	10 (10.53%)	
Strasbourg (France)	58 (20.94%)	14 (14.74%)	
Nancy (France)	53 (19.13%)	18 (18.95%)	
Dijon (France)	73 (26.35%)	28 (29.47%)	
Liege (Begium)	58 (20.94%)	25 (26.31%)	

Bold values indicate statistically significant differences.

others (115/372, 30.9%) received 5 to 6 weeks of radiotherapy alone (45–59.4 Gy). The remaining patients (10/372, 2.7%) underwent primary surgery [local excision ($n = 9$) or abdominoperineal resection ($n = 1$)], most often (9/10, 90%) without neoadjuvant therapy. For these patients, the cTNM was concordant with the pTNM.

Study population: clinicopathological characterization

All clinicopathological variables assessed in this study as well as the overall results are shown in Fig. 1. Explaining the diffuse $p16^{ink4a}$ (surrogate biomarker for HPV infection) immunoreactivity displayed by the large majority of tumors (340/372, 91.4%), carcinogenic HPV infection was detected by both genotyping assay (INNO-LiPA or Abbott RealTime) and in situ hybridization in 91% (323/355) of tumor specimens. The concordance between high-risk HPV status and $p16^{ink4a}$ immunoreactivity was excellent (349/355, 98.3%). HPV16 was by far the most prevalent type (301/323, 93.2%) and multiple infections were relatively rare (23/323, 7.1%). All 17 (17/372, 4.6%) non-interpretable samples were fixed in Bouin's fluid. Largely correlated with mutated/deleted *TP53* [24], aberrant p53 expression (undetectable or ≥50% positive cells) was observed in a minority of neoplasms (39/372, 10.5%) but, importantly, was more frequently noticed in HPV/$p16^{ink4a}$-negative tumors (11/32, 34.4%) compared to their HPV-related counterparts (28/323, 8.7%). In most tumors (266/372, 71.5%), the percentage of proliferating (Ki67-positive) cells exceeded 50% and well to moderately differentiated cancers represented over two thirds (255/372, 68.5%) of specimens. Extremely specific and sensitive for categorizing anal SCC into two groups according to their cellular origin [15], semi-quantitative analysis of CK7 expression allowed to determine that neoplasms originating in the external non-keratinizing squamous mucosa (CK7 negative) were twice as frequent than tumors arising from the transitional zone (CK7 positive) (249/372, 66.9% vs. 123/372, 33.1%). Apart from 6 cases (6/32, 18.8%), all HPV-uninfected cancers (26/32, 81.2%) stained negative for CK7, in keeping with their development from the lower (squamous) part of the anal canal. Despite considerable interindividual variations, overall, high intratumoral densities of both $CD4^+$ (median: 359.2 cells/mm^2) and $CD8^+$ (median: 421.1 cells/mm^2) T cells were observed. As expected, the sum of these latter parameters was close to the absolute $CD3^+$ T-cell count (median: 839.2 cells/mm^2) determined in each sample, validating our computerized quantifications. PD-1^+ cells (median: 66.8 cells/mm^2) were less abundant within tumor microenvironment.

Risk factors predicting disease progression/recurrence

The median follow-up time was 53.8 months (range: 1.1–254 months). After completion of treatment, local recurrence/progression (in case of residual disease) occurred in 53 (53/372, 14.2%) patients and metastatic disease was diagnosed in 30 (30/372, 8.1%) patients. Twelve (12/372, 3.2%) patients experienced both local and distant recurrences. Among these latter, with the exception of 1 patient

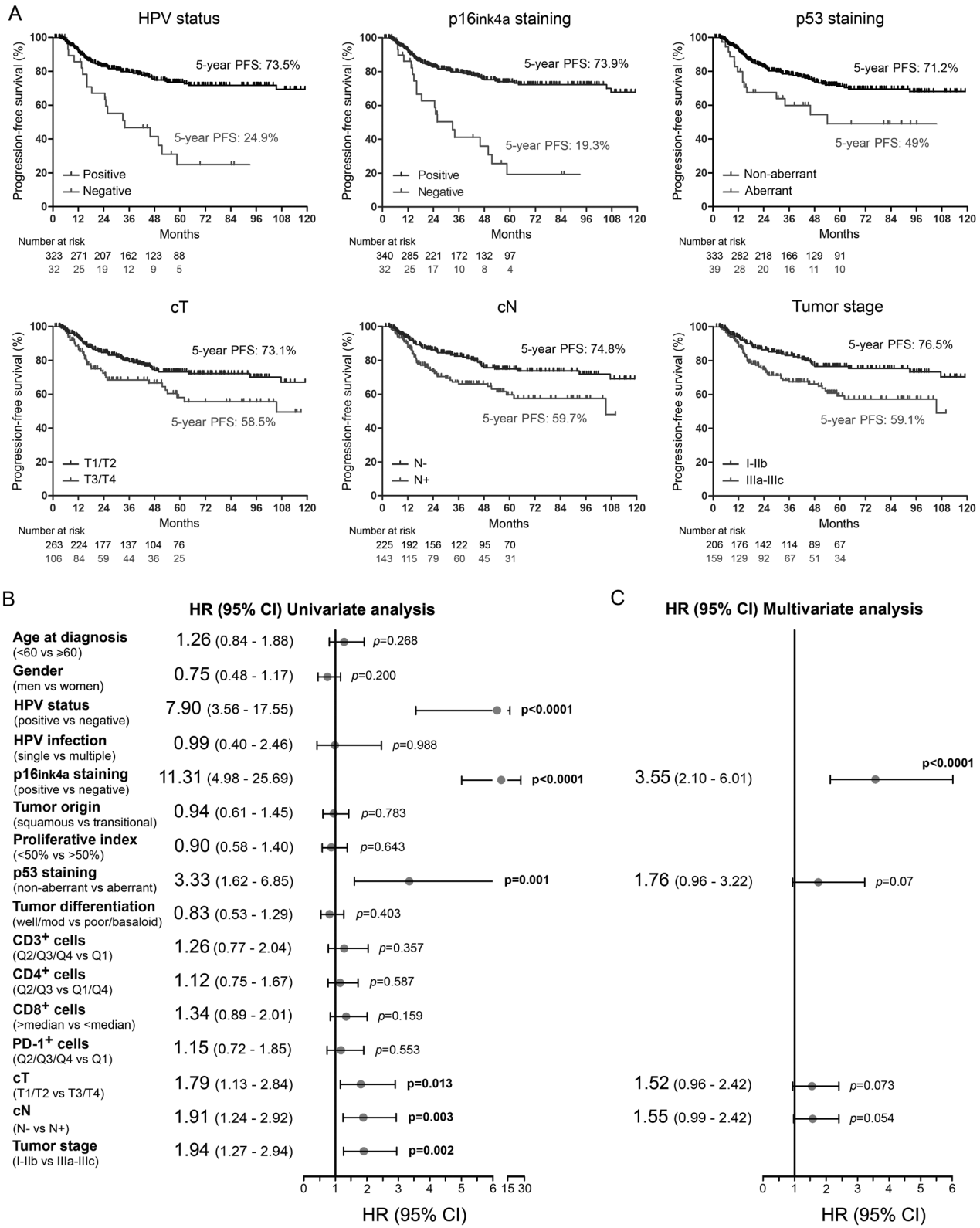

Fig. 2 Analysis of prognostic factors associated with progression-free survival. a Kaplan–Meier estimates of PFS according to significant risk factors highlighted in univariate analysis. Prognostic value of clinicopathological variables in univariate (**b**) and multivariate (**c**) analysis.

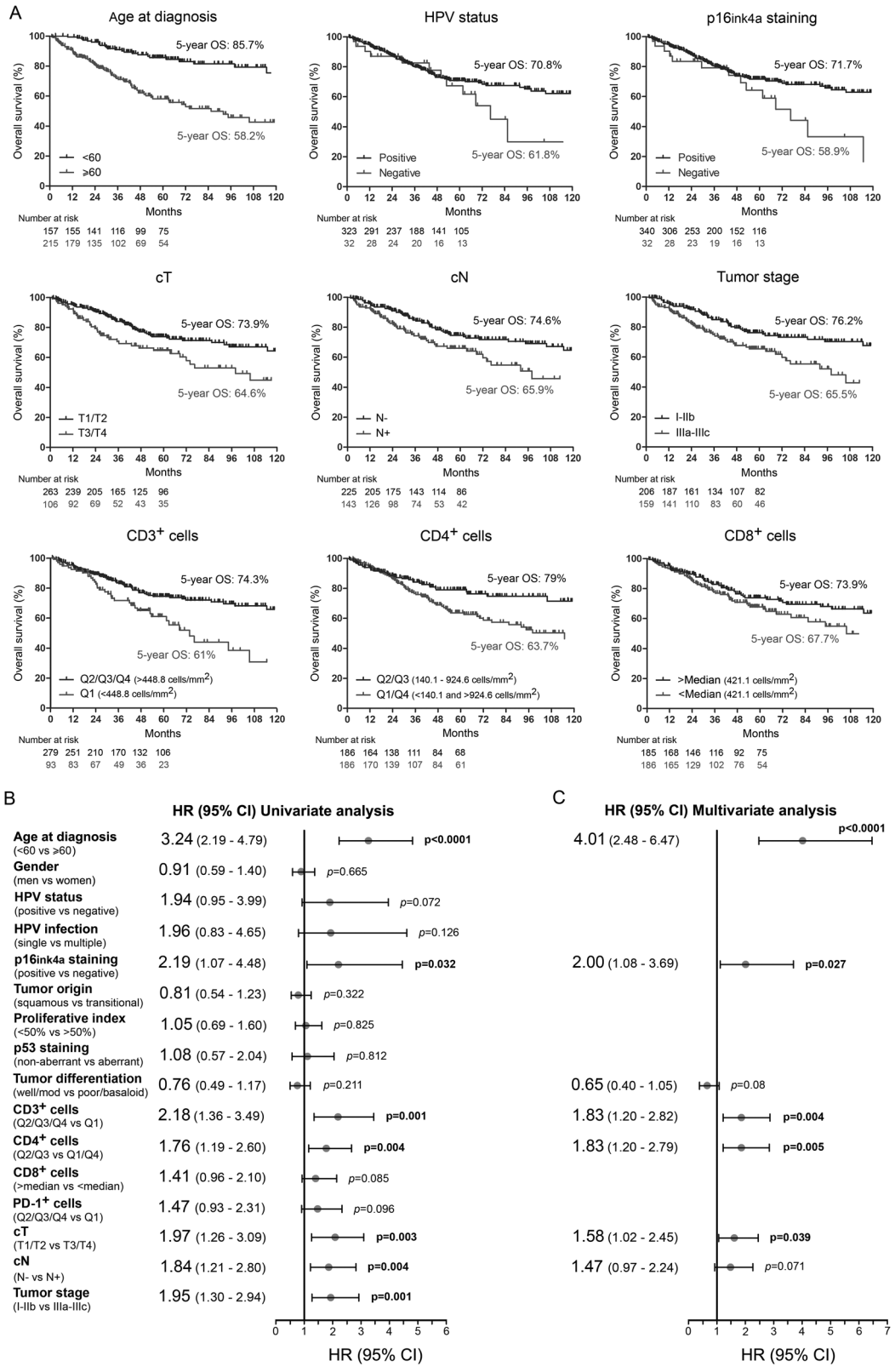

Fig. 3 Analysis of prognostic factors associated with overall survival. a Kaplan–Meier OS curves according to significant risk factors reported in univariate analysis (cut-off: $p < 0.1$). Prognostic value of clinicopathological variables in univariate (**b**) and multivariate (**c**) analysis.

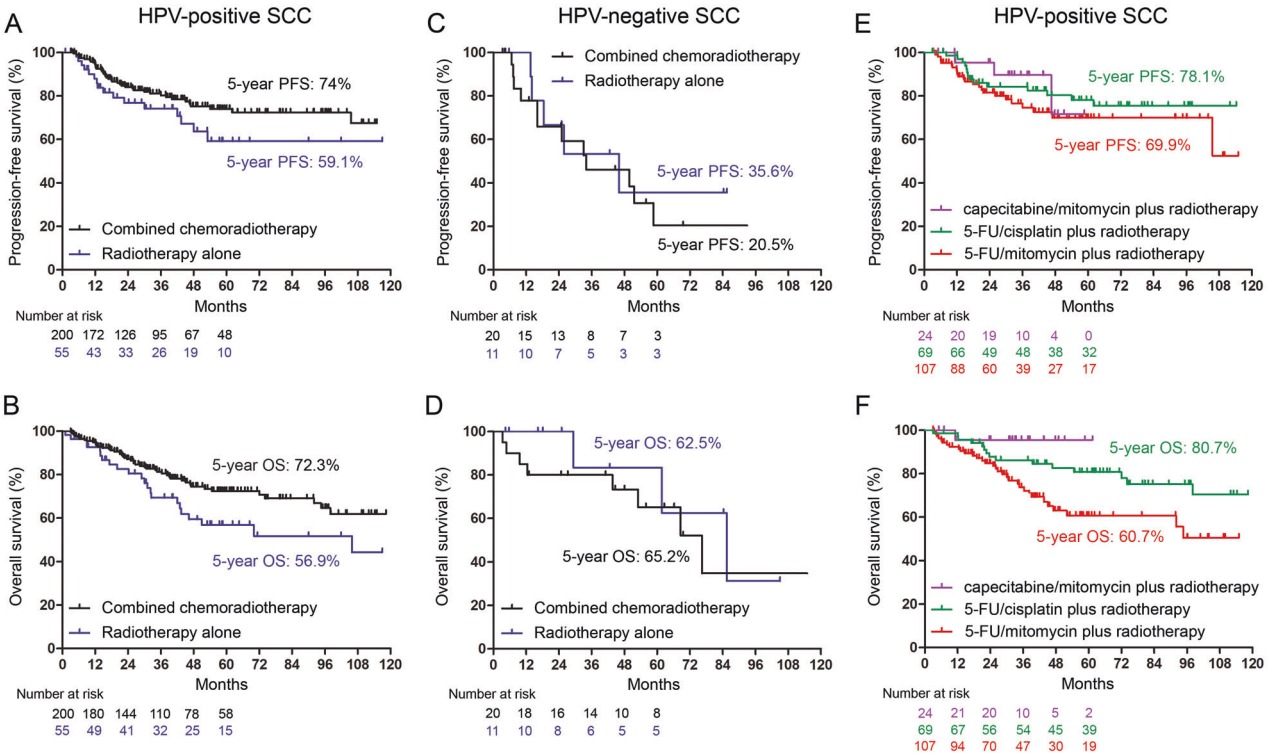

Fig. 4 Comparative efficacy of different treatment regimens. a, **c**, **e** PFS and **b**, **d**, **f** OS of patients with locally advanced (stage II-III) anal cancer according to treatment algorithms. All patients were subdivided into two groups based on their HPV status (HPV-positive vs. HPV-negative).

(1/12, 8.3%), locoregional relapse always preceded the detection of metastases (mostly in liver, lungs, and bone). The median time to local recurrence/progression and distant metastasis was 21.8 months and 25.9 months, respectively. Patient characteristics according to disease recurrence/progression are listed in Table 1. No statistical difference was noticed between the two groups in regard to age at diagnosis ($p = 0.718$), gender ($p = 0.236$), tumor origin (based on CK7 staining) ($p = 0.801$), proliferative index ($p = 0.182$), differentiation ($p = 0.597$), T-cell density ($CD3^+$, $p = 0.895$; $CD4^+$, $p = 0.556$; $CD8^+$, $p = 0.792$, $PD\text{-}1^+$, $p = 0.966$), primary treatment ($p = 0.98$) or hospital center where patients were treated ($p = 0.585$). In contrast, progressive or recurrent tumors were more often HPV/$p16^{ink4a}$-negative ($p < 0.0001$) with an aberrant p53 expression pattern ($p = 0.011$). In addition, local/distant recurrences occurred more frequently in patients diagnosed with neoplasms more than 5 cm wide ($p = 0.047$) manifesting an original lymphatic node involvement ($p = 0.029$) and, overall, in case of higher tumor stages ($p = 0.057$). Kaplan–Meier survival curves as well as both univariate and multivariate analyses for PFS are shown in Fig. 2. As opposed to 73.5% for HPV/$p16^{ink4a}$-positive cancers, importantly, 5-year PFS was only 24.9% and 19.3% for patients with HPV-uninfected [HR = 7.90; $p < 0.0001$] and $p16^{ink4a}$-negative (HR = 11.31; $p < 0.0001$) tumors, respectively. In univariate analysis, aberrant p53 staining (HR = 3.33; $p = 0.001$), high T-classification (T3/T4) (HR = 1.79; $p = 0.013$), lymph node positivity (HR = 1.91; $p = 0.003$) and advanced tumor stage (IIIa-IIIc) (HR = 1.94; $p = 0.002$) were also statistically related to poorer PFS. As shown in Fig. 2c, the multivariate analysis indicated that $p16^{ink4a}$ negative status ($p < 0.0001$) was a strong independent predictor for reduced PFS.

Prognostic factors for overall survival

During the follow-up period, 102 out of 372 (27.4%) patients died as a result of anal SCC or other causes (most notably old age). The OS (Kaplan–Meier curves) and the results of both univariate and multivariate analyses are shown in Fig. 3. In univariate analysis, the following clinicopathological factors were significantly associated with decreased OS: age at diagnosis (over 60) (HR = 3.24; $p < 0.0001$), $p16^{ink4a}$ negativity (HR = 2.19; $p = 0.032$), low intratumoral $CD3^+$ T-cell density (HR = 2.18; $p = 0.001$), low/high $CD4^+$ T-cell count (hormetic effect, Supplementary Fig. 1) (HR = 1.76; $p = 0.004$), high T-classification (T3/T4) (HR = 1.97; $p = 0.003$), positive nodal status (HR = 1.84; $p = 0.004$) and advanced tumor stage (IIIa-IIIc) (HR = 1.95; $p = 0.001$). Although patients with HPV-negative cancer tended to have a poorer OS compared to those treated for a HPV-positive tumor,

statistical significance was not reached (HR = 1.94; $p = 0.072$). A similar tendency was also reported in case of low densities of $CD8^+$ (HR = 1.41; $p = 0.085$) and $PD\text{-}1^+$ (HR = 1.47; $p = 0.096$) T cells within tumor microenvironment. As shown in Fig. 3c, patient age ($p < 0.0001$), $p16^{ink4a}$ negativity ($p = 0.027$), tumor size ($p = 0.039$) as well as both intratumoral $CD3^+$ ($p = 0.004$) and $CD4^+$ ($p = 0.005$) T-cell infiltrations were identified as independent prognostic factors for OS by multivariate analysis (Fig. 3c).

Efficacy of different treatment regimens

Out of 247 patients treated with chemoradiotherapy, 5-FU (750–1000 mg/m^2 on days 1–5 and 29–32)/mitomycin (10–12 mg/m^2 on day 1) was the predominantly used chemotherapeutic combination (133/247, 53.8%) followed by 5-FU/cisplatin (60–80 mg/m^2 on days 1 and 29 or 25 mg/m^2 weekly) (81/247, 32.8%) and capecitabine (2 × 825 mg/m^2 on radiation days)/mitomycin (25/247, 10.1%). The remaining patients (8/247, 3.2%) received only one anti-cancer agent or other drug cocktails such as mitomycin/cisplatin. Given the strong prognostic value of HPV status, and as routinely done with head and neck SCC for a decade [25, 26], anal cancer patients were sub-classified into two groups (HPV-positive vs. HPV-negative) and the efficacy of different combination drugs was assessed. As shown in Fig. 4a, b, combined chemoradiotherapy was superior to radiotherapy alone in the treatment of locally advanced (stage II-III) HPV-positive anal canal SCC [5-year PFS: 74% vs. 59.1% ($p = 0.084$); 5-year OS: 72.3% vs. 56.9% ($p = 0.089$)]. In contrast, although the total number of patients was relatively low, no benefit from the addition of chemotherapy to concurrent radiotherapy was observed with HPV-negative cancers and 5-year PFS was under 40% whatever the treatment administered (Fig. 4c). The efficacy of each individual chemoradiotherapy regimen was then assessed in the HPV-positive group. Evidence of improvement in both 5-year PFS (78.1% vs. 69.9%, $p = 0.201$) and OS (80.7% vs. 60.7%, $p = 0.024$) was observed with 5-FU/cisplatin compared to 5-FU/mitomycin chemoradiotherapy. As detailed in Supplementary Table 1, these latter results are unlikely to be explicated by differences in term of patient age, gender, HPV genotypes, tumor origin, proliferative index, p53 status, differentiation or cTNM/tumor stage classification. The low number of patients treated with capecitabine/mitomycin combined with radiotherapy does not allow to draw any conclusion regarding this treatment regimen.

Discussion

Based on a better understanding of cancer pathobiology, both the diagnosis and the subsequent management of most liquid and solid tumors have considerably evolved over the past decade, allowing a global improvement of patient outcome and quality of life. Although its incidence is increasing by 2–3% annually in high-income countries [2], anal carcinoma represents undoubtedly an exception. Indeed, treatment is still largely (if not exclusively) dictated by tumor staging and concurrent chemoradiotherapy has remained the gold standard of care for most (>90%) patients for almost 30 years [27]. This current situation is mainly explained by controversial results reported by earlier single-institution studies with limited sample sizes and/or containing poorly described patients with uncontrolled HIV infection (before the availability of highly active antiretroviral drugs). Aiming at in depth characterizing SCC arising from the anal canal, the present large multicenter cohort was first collected and the prognostic significance of various clinicopathological variables was explored. In agreement with half a dozen smaller studies published in the past few years [13–15, 28–31], HPV/$p16^{ink4a}$-negative status has been clearly shown as a robust predictor for unfavorable patient outcome. Similarly, aberrant p53 expression was also associated with poor response to treatment. Of note, in a weak proportion (<10%) of tumors, HPV infection and mutated *TP53* coexisted and, importantly, these latter neoplasms were associated with an intermediate prognosis [5-year PFS: 64.5% vs. 74.8% ($p = 0.048$)] (Fig. 5). Also mentioned in the context of both cervical and oropharyngeal cancers [32–34], these data further support the predictive value of *TP53* status in cancer patients undergoing chemoradiation-based therapy. Although no link between tumor origin and patient survival was observed in this larger study, the 2:1 ratio between the two region-specific subtypes of anal SCC (squamous zone vs. anal transitional zone) as well as the higher proportion of

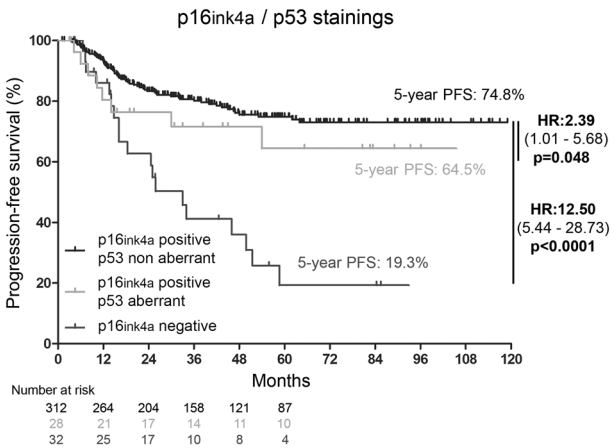

Fig. 5 Progression-free survival of anal cancer patients according to p16ink4a/p53 status. All patients were subdivided into three groups (p16ink4a-positive/p53 non aberrant vs. p16ink4a-positive/p53 aberrant vs. p16ink4a-negative). The intermediate prognosis of HPV-driven neoplasms displaying a mutated TP53 should be noticed.

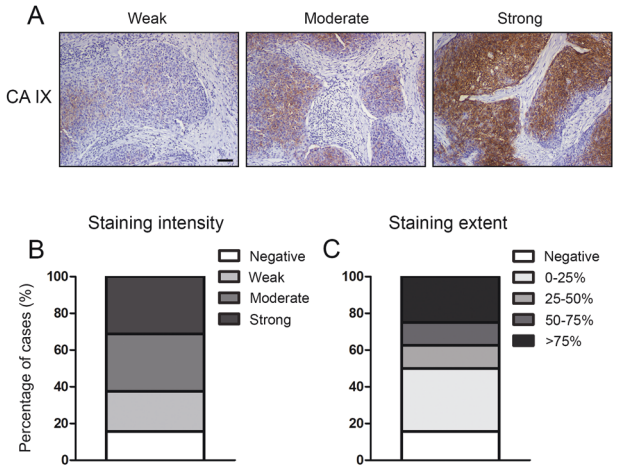

Fig. 6 Carbonic anhydrase IX, an endogenous hypoxia biomarker, expression in HPV-negative anal SCC. a Representative examples of anal cancer stained for CA IX. Semi-quantitative analysis of CA IX staining intensity (**b**) and extent (**c**) displayed by HPV-negative anal SCC ($n = 32$). The scale bar represents 100 µm.

HPV-uninfected neoplasms detected in the external part of the anal canal were confirmed. Interestingly, this latter result closely mimics the reported HPV prevalence for gynecological SCC (virtually all cervical cancers are HPV-positive whereas only about 75–80% vaginal neoplasms are etiologically related to HPV infection) [35].

Although never previously analyzed in anal cancers, the high densities of T-cell subsets within tumor microenvironment as well as the identification of $CD3^+$, $CD8^+$, and $PD\text{-}1^+$ tumor-infiltrating lymphocytes as favorable prognostic biomarkers were not really surprising given recent reports focusing on oropharynx [17–19]. The unexpected finding was the clear-cut beneficial effect of moderate densities of $CD4^+$ cells (Supplementary Fig. 1). Whether this hormetic effect is related to differences in T cell polarization (e.g., Th1, Th2, Th17, Treg) is unknown but, in view of its substantial prognostic value, single-cell RNA sequencing or flow cytometry analysis using enzymatically-dissociated fresh cancer biopsies certainly represent interesting perspectives.

Another striking finding in this study was the very high risk of disease progression and/or local/distant recurrence in patients with HPV-negative cancer, whatever the treatment regimen administered (chemoradiotherapy or radiotherapy alone). As commonly considered with oropharyngeal or vulvar tumors [25, 26], HPV-positive anal cancers and their HPV-negative counterparts represent undeniably two distinct entities and should, therefore, not be treated in the same way. The present data argues for a low radiosensitivity of HPV-uninfected SCC and, for lack of anything better, surgical resection could provide better outcomes. The use of hypoxia-activated pro-drugs (e.g., Nimorazole and Tirapazamine) alongside chemoradiotherapy could also be regarded as an attractive alternative [36]. Currently tested with head and neck SCC patients (NCT01950689), targeting hypoxia therapeutically might be efficient given that 27 out of 32 (84.4%) HPV-negative anal cancers displayed hypoxic areas, as supported by the expression of the endogenous hypoxia biomarker CA IX (Fig. 6).

The chemotherapeutic drug combination used as first line regimen for HPV-positive patients has been a matter of debate for a long time and is still mainly dependent on clinician preferences. Here, we showed that chemoradiation using 5-FU and cisplatin was superior to concurrent chemoradiotherapy involving 5-FU/mitomycin. Although our study is limited by its retrospective design and the toxicity profile between groups was not assessed, the present results intriguingly confirm the clinical success of cisplatin in the treatment of HPV-positive tumors. Indeed, cisplatin demonstrated clear benefits in terms of overall survival and reduced recurrences compared to other common chemotherapy regimens or targeted therapies (e.g., cetuximab) for both cervical and HPV-positive oropharyngeal cancer patients [37–39]. Although still not fully understood, recent evidences demonstrated that DNA damage pathways are hijacked by HPV E6/E7 oncoproteins to promote viral life cycle [40, 41]. Therefore, this latter phenomenon could represent the Achilles' heel of HPV-positive cancers and explain their high sensitivity to cisplatin-induced DNA single/double-strand breaks.

In conclusion, traditional prognostic factors such as tumor size and nodal status as well as treatment algorithms used since the mid-90's for non-metastatic anal SCC have clearly reached their limits. Based on both the present information and the literature on oropharyngeal, vaginal/vulvar and penile cancers accumulated during the last decade, anal cancer patients should be systematically separated into two groups according to HPV/$p16^{ink4a}$ status. In case of *TP53* mutation in HPV-driven cancer, a closer monitoring could be considered. The potential therapeutic benefit of cisplatin-based chemoradiotherapy or immune checkpoint inhibitors (e.g., anti-PD-1) compared to concurrent radiotherapy plus 5-FU and mitomycin would require further investigations. Finally, our results point to the crucial requirement to optimize current treatment and/or to investigate novel drugs/strategies for treating HPV-negative anal cancers.

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Author contributions MH designed the study; FM, PR, LV, ALe, ALu, PM, LA, M-CB-L, LD, LM, DP, AL, J-BD, J-PG, A-SW, PD, J-FB, and SV-D collected data/tissue specimens; DB, PR, EH, CP, TL, CR, MA, J-LP, and MH performed experiments; DB, FM, PC, ChlM, PH, PD, ChrM, SV-D, and MH interpreted the data and/or reviewed the samples; DB, OP, and MH performed the statistical analysis; DB and MH generated the Figures; MH wrote the paper. All authors had final approval of the submitted manuscript.

Compliance with ethical standards

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

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