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Original Research

Prognostic value of high-risk human papillomavirus DNA and p16^{INK4a} immunohistochemistry in patients with anal cancer: An individual patient data meta-analysis

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KEYWORDS
ASCC;
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Meta-analysis;
IPD

Abstract ***Background:*** High-risk human papillomavirus (hrHPV) types represent the aetiological agents in a major proportion of anal squamous cell carcinomas (ASCC). Several studies have suggested a prognostic relevance of HPV-related markers, particularly hrHPV DNA and $p16^{INK4a}$ (p16) protein expression, in patients with ASCC. However, broader evaluation of these prognostic marker candidates has been hampered by small cohort sizes and heterogeneous survival data among the individual studies. We conducted an individual patient data (IPD) meta-analysis to determine the prognostic value of hrHPV DNA and p16 in patients with ASCC while controlling for major clinical and tumour covariates.

Patients and methods: A systematic literature search was conducted to identify all published studies analysing p16 alone or in combination with hrHPV DNA and reporting survival data in patients with ASCC. Clinical and tumour-related IPD were requested from authors of potentially eligible studies. Survival analyses were performed with a proportional hazard Cox model stratified by study and adjusted for relevant covariates. The study-specific hazard ratios (HRs) for the exposures were pooled using a random-effects model. Kaplan-Meier curves from different studies were pooled per exposure group and weighted by the study's total sample size.

Results: Seven studies providing IPD from 693 patients with ASCC could be included in the meta-analysis. Seventy-six percent of patients were p16+/hrHPV DNA+, whereas 11% were negative for both markers. A discordant marker status was observed in 13% of cases. Patients with p16+/hrHPV DNA+ ASCC showed significantly superior overall survival (OS) compared with patients with p16-/hrHPV DNA- tumours (pooled adjusted HR = 0.26 [95% confidence interval {CI}, 0.14–0.50]) with pooled three-year OS rates of 86% (95% CI, 82–90%) versus 39% (95% CI, 24–54%). Patients with discordant p16 and hrHPV DNA status showed intermediate three-year OS rates (75% [95% CI, 56–86%] for p16+/hrHPV DNA- and 55% [95% CI, 35–71%] for p16-/hrHPV DNA+ ASCC).

Conclusion: This first IPD meta-analysis controlling for confounding variables shows that patients with p16+/hrHPV DNA+ ASCC have a significantly better survival than patients with p16-/hrHPV DNA- tumours.

1. Introduction

Anal cancer represents about 2.5% of all gastrointestinal malignancies [1]. Among these cases, anal squamous cell carcinoma (ASCC) is by far the most common histological subtype, accounting for more than 80% of anal cancer cases [2,3]. Notably, the incidence of anal cancer has significantly increased worldwide in the past decades [4,5], with more than 48,000 new cases estimated for 2018 [6]. It has been suggested that this rising incidence is related to altered sexual behaviour associated with an increased risk of acquiring an infection with oncogenic human papillomaviruses (HPV) [5]. Oncogenic, that is high-risk (hr), HPV types represent the major aetiological factor of anal cancer development. A recent study by de Martel et al. [7] estimated HPV to account for 88% of all anal cancer cases. Further well-known risk factors of anal cancer development, such as receptive anal intercourse, iatrogenic or human immunodeficiency virus (HIV)-induced immune suppression, smoking and genital dysplasia in women, most likely promote carcinogenesis by increasing the probability of hrHPV persistence and subsequent hrHPV-induced transformation [8–10].

HrHPV can cause premalignant and malignant lesions in other anogenital sites, including the uterine cervix, vulva, vagina and penis, as well as a proportion of cancers in the oropharynx [7,11,12]. Comprehensive studies of the past decades have unveiled common molecular events that govern HPV-induced carcinogenesis in those sites. It is well established to date that both initiation and maintenance of hrHPV-induced transformation critically depend on sustained expression of the hrHPV E6 and E7 oncoproteins that deregulate numerous physiological processes in the cell [13,14]. Consequently, a tumour is considered to be causally driven by hrHPV, if E6/E7 overexpression can be demonstrated. However, detection of hrHPV E6/E7 gene products (i.e. mRNA or proteins) from tumour tissue is not easily feasible in all routine diagnostic laboratories. Thus, surrogate markers of a transforming hrHPV infection, for example the presence of hrHPV DNA or overexpression of $p16^{INK4a}$, represent viable

alternatives in this setting, albeit coming along with some individual drawbacks. As such, hrHPV DNA may principally also be detected in non-transforming HPV infections (reviewed in [15,16]) and therefore overestimate the proportion of truly HPV-driven tumours. $p16^{INK4a}$ is a cellular protein that becomes considerably overexpressed by hrHPV E7 oncoprotein signalling, thus indicating transforming activity of hrHPV [17]. Detection of $p16^{INK4a}$ overexpression by immunochemistry is now commonly used in the diagnosis of HPV-transformed lesions at the uterine cervix and in the triage of screen-positive women [18,19]. However, $p16^{INK4a}$ overexpression also occurs in the absence of HPV infection in about 5–15% of tumours [20–22]. To increase the specificity with minimal loss in sensitivity, it has been suggested to combine hrHPV DNA and $p16^{INK4a}$ testing for the reliable identification of HPV-induced tumours in different sites of the genital and head and neck region [20,23,24].

Patients with hrHPV-related cancers generally show improved survival compared with patients with HPV-negative cancers in the same locations. This observation has been confirmed in several meta-analyses for cancers arising from the vulva, the penis and the oropharynx [25–29]. Classification of the HPV status mostly relied on the detection of HPV DNA and/or $p16^{INK4a}$ overexpression in those studies. Two recent meta-analyses have also demonstrated improved survival for patients with HPV-driven compared with HPV-negative anal cancer [30,31]. However, those meta-analyses were based on aggregated study data where control of confounding effects is often problematic because of insufficient and/or heterogeneous documentation. This precludes the assessment of important variables, such as age, gender or tumour status, that are known to impact survival.

We conducted for the first time an individual patient data (IPD) meta-analysis on the prognostic value of HPV in patients with anal cancer, taking into consideration multiple clinical and tumour covariates. To account for the limited individual accuracy of $p16^{INK4a}$ and hrHPV DNA to identify HPV-induced cancers, we compared the prognostic impact of these surrogate markers alone and in combination. We further determined survival of patients with discordant results on $p16^{INK4a}$ and hrHPV DNA testing (i.e. $p16^{INK4a}$-negative/hrHPV DNA-positive or $p16^{INK4a}$-positive/hrHPV DNA-negative cases) as these subgroups are hypothesised to represent biologically and clinically distinct tumour entities compared with truly HPV-driven or HPV-negative anal cancers.

2. Methods

2.1. Clinical question

The aim of this IPD meta-analysis was to investigate whether $p16^{INK4a}$ overexpression (abbreviated in this article as p16) assessed by immunohistochemistry (IHC) can serve as a prognostic marker of overall survival (OS) and how it compares with hrHPV DNA detection and a combination of p16 IHC and hrHPV DNA. The clinical question was disentangled in the following PICOS (population–intervention/index test–comparator tests–outcomes–study design) scheme (Table 1).

2.2. Data

A broad search string was defined to conduct a systematic search on the National Center for Biotechnology Information PubMed database: '(p16*) AND (Anal OR Anus OR ASCC OR AIN OR A-SCC OR ASIL)'. The following inclusion criteria were adopted: provision of original data, conduction of p16 IHC on anal cancer specimens, analysed sample number $\geq$10, and availability of patient survival data. No restrictions regarding language were applied. In case study data from a distinct patient cohort were presented in more than one report, only the one providing the most comprehensive study data (as per the inclusion criteria) was included in the meta-analysis. The authors of all studies found to be potentially eligible during the screening process were contacted with specifically designed data forms to obtain the following IPD: gender, age at diagnosis, classification of the anatomic extent of the tumor disease as per the TNM Classification of Malignant Tumours (TNM), p16 status, hrHPV DNA status, HIV status, localisation of the tumour, therapy administered and disease progression and vital status as per the calendar date. At least two attempts

Table 1
PICOS scheme.

Component	Specification
Population	Men or women with a diagnosis of anal cancer Confounding variables: gender, age, TNM stage, HIV status, therapy
Intervention	p16 IHC staining of anal cancer tissue sections
Comparator	C1: hrHPV DNA C2: hrHPV DNA and p16 IHC combined
Outcomes	Differences in overall survival after distinct time intervals (e.g. after 36 months, 60 months etc.) between test-positive (+) and test-negative (−) groups
Study design	Cohort studies (prospective or retrospective) or randomised controlled trials providing data on the p16 IHC expression status at diagnosis and patient survival

IHC, immunohistochemistry; hrHPV, high-risk human papillomavirus; HIV, human immunodeficiency virus.

were carried out to establish contact with authors or co-authors of eligible published reports. If authors did not respond or could not provide the required data, the respective studies were excluded from further analyses. In addition, authors were asked to provide definitions of p16 positivity, OS and progression-free survival, if these had not been specifically explained in the article. For the present study, we assessed only OS (summarised hereafter as 'survival').

2.3. Study selection

The selection process of eligible studies is illustrated in the PRISMA flow diagram (Fig. 1). Overall, 207 articles were identified with the designed search string during the last search on 13th June 2020. Nineteen of those studies met all inclusion criteria and were deemed potentially eligible for inclusion. Authors of eight studies who published between 2011 and 2017 provided the requested IPD [32–39]. One study had to be excluded because the authors could only provide data from p16-positive patients precluding comparison with p16-negative patients [39]. In the final IPD meta-analysis, 693 patients with anal cancer of seven eligible studies could be included [32–38]. Only studies with a predetermined cut-off for hrHPV DNA-positivity and clearly defined p16-positivity status were included in the main analysis; one study [37] only had data on the p16 status and was not included in any hrHPV DNA or p16/hrHPV DNA analysis.

2.4. Statistical analysis

The assessed exposure variables were defined in terms of p16 status, hrHPV DNA status and the interaction between both. A proportional hazard Cox model was fitted that was stratified by study to assess differences in survival by exposure and was adjusted or not for the following covariates: age, gender, N-stage and T-stage. Age was

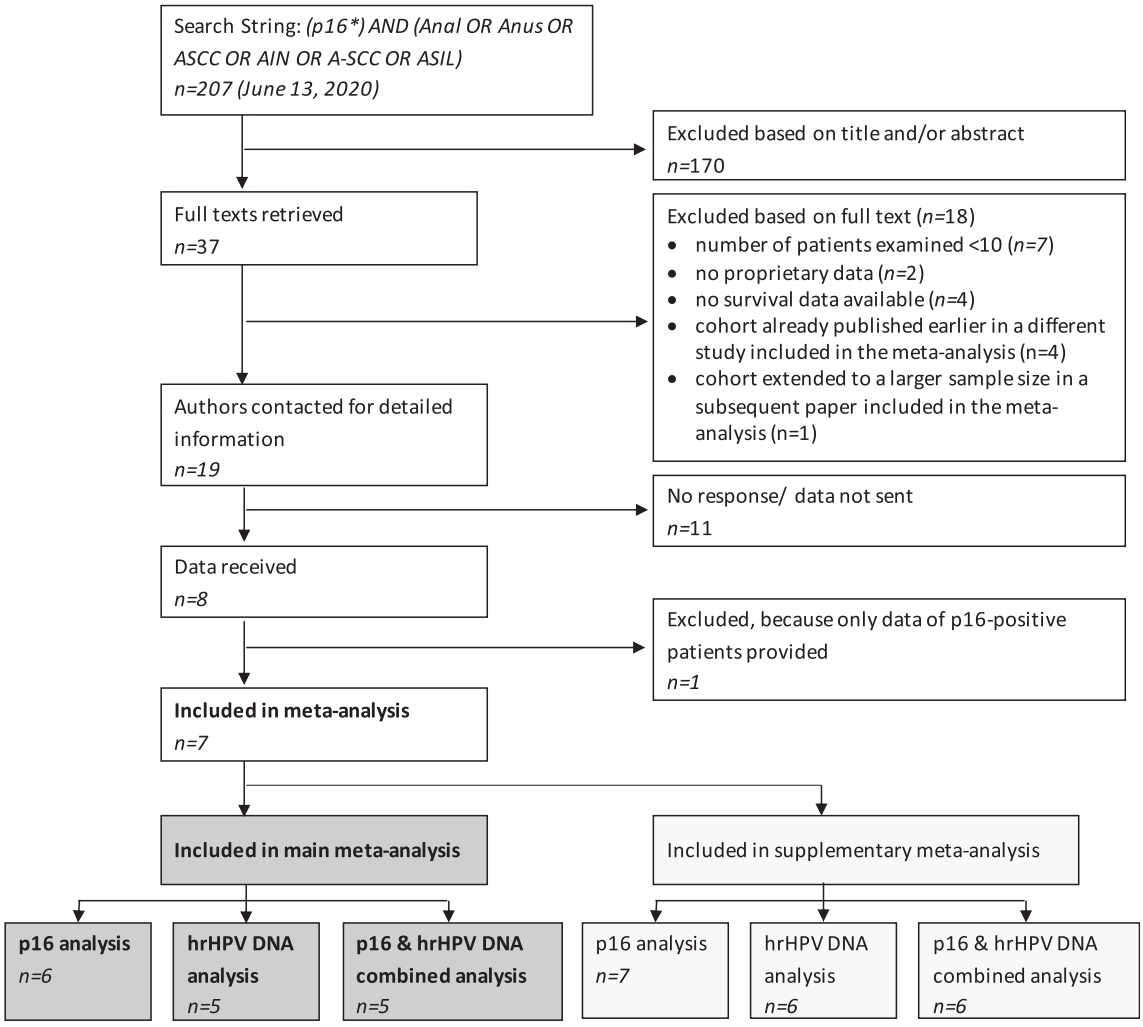

Fig. 1. PRISMA flow diagram.

Table 2
Patient characteristics among the seven studies included in the meta-analysis.

Characteristics	Yhim, 2011	Gilbert, 2013	Koerber, 2014	Mai, 2015	Meulendijks, 2015	Balermpas, 2017	Belgioia, 2015	Total
Period of patient inclusion	1998–2009	2004–2009	2000–2011	1990–2012	2003–2011	1989–2016	2009–2014	
Follow-up duration: median and range (months)	39 (2–111)	28 (1–87)	49 (3–169)	47 (1–205)	32 (3–96)	40 (1–325)	26 (1–71)	35 (1–325)
Cohort size (n)	47	153	90	106	106	150	41	693
Age: median and range (years)	65 (44–90)	62 (34–93)	55 (22–94)	60 (31–86)	60 (34–86)	59 (30–84)	63 (32–84)	61 (22–94)
Gender								
Female	25 (53%)	93 (61%)	77 (86%)	63 (59%)	56 (53%)	84 (56%)	38 (93%)	436 (63%)
Male	22 (47%)	60 (39%)	13 (14%)	43 (41%)	50 (47%)	66 (44%)	3 (7%)	257 (37%)
T-stage								
T1/T2	31 (66%)	59 (39%)	64 (71%)	79 (75%)	56 (53%)	106 (71%)	22 (54%)	417 (60%)
T3/T4	16 (34%)	65 (42%)	26 (29%)	27 (25%)	50 (47%)	44 (29%)	19 (46%)	247 (36%)
N/A		29 (19%)						29 (4%)
N-stage								
N0	29 (62%)	80 (52%)	68 (76%)	70 (66%)	48 (45%)	99 (66%)	16 (39%)	410 (59%)
N1–3	18 (38%)	53 (35%)	22 (24%)	36 (34%)	57 (54%)	51 (34%)	25 (61%)	262 (38%)
N/A		20 (13%)			1 (1%)			21 (3%)
UICC stage (7th ed.)								
Stage I		9 (6%)	15 (17%)	16 (15%)	4 (4%)		2 (5%)	56 (8%)
Stage II		52 (34%)	46 (51%)	45 (43%)	36 (34%)		12 (29%)	191 (28%)
Stage IIIA		24 (16%)	11 (12%)	11 (10%)	34 (32%)		12 (29%)	92 (13%)
Stage IIIB		37 (24%)	16 (18%)	20 (19%)	31 (29%)		14 (34%)	118 (17%)
Stage IV		3 (2%)	2 (2%)	13 (12%)				18 (3%)
N/A	47 (100%)	28 (18%)		1 (1%)	1 (1%)	150 (100%)	1 (2%)	228 (33%)
HIV status								
Positive		9 (6%)	1 (1%)	2 (2%)	10 (9%)	24 (16%)	2 (5%)	48 (7%)
Negative	47 (100%)	70 (46%)	81 (90%)		34 (32%)	126 (84%)	39 (95%)	397 (57%)
N/A		74 (48%)	8 (9%)	104 (98%)	62 (59%)			248 (36%)

entered in the model as a continuous variable, whereas the other covariates were entered as binary variables. Tumour stages were grouped as T1/T2 versus T3/T4, and nodal stages were grouped as N0 versus N1/N2/N3. We assumed a common effect for age, gender, N-stage and T-stage over all the studies, whereas we allowed the effect of the exposure variables to differ among studies. A random-effects (RE) model was applied for pooling the hazard ratios (HRs) using the DerSimonian-Laird estimator with the metafor package in R [40]. The analysis was performed on the log(HR) scale with log-log-based standard errors. The percentage of total variation due to inter-study heterogeneity was assessed by the I^2 index [41]. The log(HR) values were eventually back-transformed to HRs and shown in forest plots.

Kaplan-Meier (KM) curves from different studies were pooled per exposure group, weighted by the study's total sample size [42]. The maximum follow-up time was different between exposure groups and studies. Therefore, in the KM, the maximum time analysed for each exposure group was determined by the study with the smallest follow-up time for that group. From these survival curves, we obtained the survival rate at three years and the median survival time, the minimum time for which the OS is at least 50%. To compare the distribution of categorical variables between groups, a chi-square test with Monte Carlo simulated p values was used, and when continuous variables between groups were compared, ANOVA was applied.

3. Results

3.1. Study, patient and tumour characteristics

Six of the seven included studies were conducted in Europe (three in Germany [32,33,38], one in the United Kingdom [36], one in Italy [37] and one in the Netherlands [34]), whereas one study was conducted in Asia (South Korea) [35]. A retrospective cohort study design was applied in all seven studies. The maximum observation time varied between 6 [37] and 27 years [38]. In the original studies, patients had been excluded for the following reasons: patients receiving palliative treatment or having recurrent disease [32], no curative intent of treatment or occurrence of previous malignancies [38], metastatic disease [36], metastatic disease or prior pelvic radiotherapy [37], no paraffin-embedded tumour tissue available [34,35], and patients with missing data [33]. In all studies, patients were treated with radio-chemotherapy, radio-chemotherapy and surgery or radiotherapy alone. Almost two-thirds of the patients were women (n = 436, 63%). The median age at the time of diagnosis ranged from 55 to 65 years. Forty-eight among 445 patients (11%) with available

information on the HIV status were known to be HIV-positive. A summary of patient characteristics is shown in Table 2. In all studies, formalin-fixed paraffin-embedded tissue samples were prepared and used for p16 and HPV DNA analyses. For one study, the cut-off for hrHPV DNA positivity was not pre-defined but based on the median of obtained test results for hrHPV 16 in the original study [38]. The reviewing authors therefore defined a cut-off for this test *a posteriori*. Consequently, this study was included in the main analysis for the results of the prognostic value of p16 but not for hrHPV DNA. However, in a sensitivity analysis, this study was included for the prognostic value of hrHPV DNA and the combination with p16 considering the *a posteriori* HPV DNA cut-off (see Supplementary Materials). Anal cancers included in the original studies were of squamous cell origin (that is ASCC), representing the most common histological type of anal cancer, therefore the terms anal cancer and ASCC are used interchangeably in this article. More detailed information of the characteristics of the included studies is provided in Supplementary Table 1.

3.1.1. p16 overexpression in patients with anal cancer

p16 IHC results were available from 687 of 693 (99%) patients. Seventy-seven percent (526/687) of the patients with anal cancer were found to be p16+, and 161 (23%) were p16- (Table 3). Of note, definitions of a positive p16 IHC test result were heterogeneous among the included studies. Some authors considered the staining intensity [36], whereas others incorporated the staining pattern [32,33] or composite scores of staining intensity and percentage of positive cells [34,38] or of staining intensity, pattern and percentage of positive tumour cells [35] into the definition. A common definition was found among two studies only [32,33]. No information on the definition of a positive test result was provided by one study [37]. Therefore, this study was excluded from the main analysis. The detailed definitions used within the included studies are provided in Supplementary Table 2. For the six studies with p16 results included in the main analysis, the average age in p16+ patients was 60.2 years and did not significantly differ from that of p16- patients with 61.2 years ($p = 0.3864$). In women, the pooled p16-positivity was 83%, significantly differing from the pooled prevalence in men (65%; $p < 0.0001$). The distribution of covariates among p16+ and p16- patients is illustrated in Supplementary Figs. 1 and 4.

3.1.2. hrHPV DNA in patients with anal cancer

Six of the seven included studies performed HPV DNA testing involving 601 tested patients [32–36,38]. All of them applied polymerase chain reaction-based methods that were able to detect hrHPV DNA types. While some of the six studies also detected low-risk HPV DNA types, our meta-analysis exclusively focused on hrHPV DNA types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 [43]). The detectable hrHPV DNA types as well as the applied techniques and cut-offs for a positive test result differed among the included studies (Supplementary Table 7). Notably, one study used a rather analytical cut-off for defining HPV DNA-positivity by determining the median of HPV 16 DNA copies of the cohort and was therefore excluded from the main analysis [38]. An *a posteriori* cut-off was determined in this meta-analysis, defining a positive test result by a viral load of ≥1 HPV 16 DNA copy/beta-globin copy. This threshold was based on the rationale that truly HPV-induced tumours should harbour at least one viral copy per tumour cell [44]. The HPV 16 DNA data after cut-off adjustment of the respective study were included in the sensitivity analysis (Supplementary Tables 9 and 10).

HrHPV DNA was detected in 376 of 459 (82%) patients with a known hrHPV DNA status of the five

Table 3
Prevalence of p16 overexpression and of hrHPV DNA among all patients with anal cancer.

		p16 status			hrHPV DNA status		
Study	Year	Positive	Negative	N/A	Positive	Negative	N/A
Yhim	2011	39 (83%)	8 (17%)	0 (0%)	35 (74%)	12 (26%)	0 (0%)
Gilbert[a]	2013	137 (90%)	16 (10%)	0 (0%)	102 (67%)	8 (5%)	43 (28%)
Koerber	2014	75 (83%)	15 (17%)	0 (0%)	75 (83%)	15 (17%)	0 (0%)
Mai	2015	74 (70%)	32 (30%)	0 (0%)	72 (68%)	34 (32%)	0 (0%)
Meulendijks	2015	96 (91%)	10 (9%)	0 (0%)	92 (87%)	14 (13%)	0 (0%)
Balermpas[b]	2017	76 (51%)	74 (49%)	0 (0%)	94[b] (63%)	48[b] (32%)	8[b] (5%)
Belgioia[c]	2015	29[c] (71%)	6[c] (15%)	6[c] (15%)	–	–	41 (100%)

hrHPV, high-risk human papillomavirus.

[a] HPV genotyping data for this cohort of patients were published separately [61].

[b] Excluded from the main meta-analysis for hrHPV DNA because no clinically relevant cut-off was defined by the authors. The prevalence of hrHPV DNA is based on an *a posteriori* cut-off for HPV 16 DNA applied by the reviewers. The meta-analysis with this *a posteriori* cut-off for the study by Balermpas *et al.*, 2017, was included in a sensitivity analysis presented in the Supplementary Materials. The eight cases considered as N/A contained other non-HPV16 types, not classifiable with the *a posteriori* HPV16 cut-off.

[c] Excluded from the main meta-analysis for p16 because no information on the definition of a positive p16 test result was provided by the authors. A meta-analysis including this study (Belgioia *et al.*, 2015) was included in a sensitivity analysis presented in the Supplementary Materials.

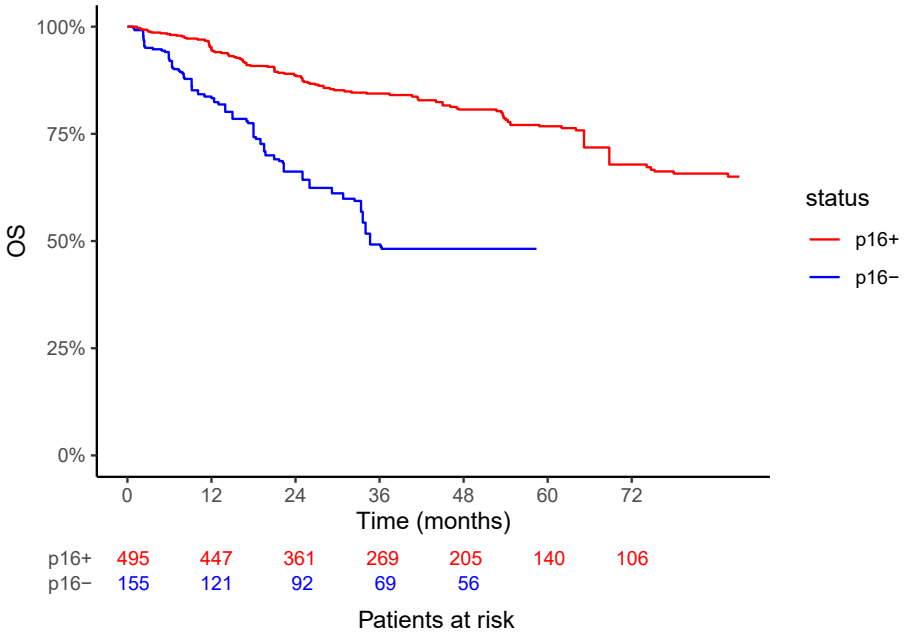

Fig. 2. Pooled overall survival (OS) curves stratified by p16 status in patients with anal cancer of the six studies included in the main analysis (for patients with a known p16 status and outcome information) and weighted by the study's total sample size. The number of patients under observation at risk of dying at the beginning of each year is mentioned under the X-axis.

studies included in the main analysis (Table 3). The most frequently observed hrHPV DNA type was HPV 16 (312 patients [85%] with a single infection and 29 patients [8%] with multiple infections). The second most common hrHPV type was HPV 18, present as single infection in 8 patients (2%) and as co-infection with other types in 11 patients (3%). Further details on hrHPV type distribution are provided in Supplementary Table 8. The percentage of hrHPV DNA+ anal cancers varied from 67% to 87% (see Table 3). The average age at diagnosis was 60.4 years and 59.6 years, in hrHPV DNA-positive and -negative cases, respectively ($p = 0.5888$). In women, hrHPV DNA-positivity was 89%, whereas it was only 71% in men ($p < 0.0001$). The distribution of covariates among hrHPV DNA+ and hrHPV DNA- patients is illustrated in Supplementary Fig. 2.

3.2. Prognostic value of the p16 status

The three-year survival rate was 84% (95% CI, 81–88%) for patients with a p16+ ASCC and 49% (95% CI, 40–58%) for patients with a p16- ASCC. A median survival time was not reached in patients with a p16+ tumour during the observation period of 87 months, whereas p16- patients showed a median survival of 35 months (Fig. 2).

Superior survival among p16+ compared with p16- patients was observed in all six studies with unadjusted HRs ranging from 0.16 (95% CI, 0.08–0.33) [36] to 0.78 (95% CI, 0.41–1.51) [38]. The pooled unadjusted HR for the six included studies was 0.39 (95% CI, 0.19–0.80) (Supplementary Fig. 6). Adjusting for age, gender, T-stage and N-stage, the pooled HR of p16+ compared with p16- patients was 0.49 (95% CI, 0.24–0.99; Fig. 3).

3.3. Prognostic value of hrHPV DNA detection

Three-year survival rates for the hrHPV DNA+ and hrHPV DNA- groups were 84% (95% CI, 80–88%) and 52% (95% CI, 38–64%), respectively. Patients with hrHPV DNA- ASCC had a median survival of 38 months, whereas median survival was not reached during the total observation period for patients with hrHPV DNA+ ASCC (Fig. 4).

The unadjusted HR for the hrHPV DNA+ compared with the hrHPV DNA- group was 0.29 (95% CI, 0.22–0.39), with HR ranging between 0.22 (95% CI, 0.07–0.69; [35]) and 0.39 (95% CI, 0.17–0.89; [33]; Supplementary Fig. 7). When adjusting for age, gender, T-stage and N-stage, the pooled HR was 0.33 (95% CI, 0.24–0.45; Fig. 5).

3.4. Combined p16 and hrHPV DNA status

The proportion of patients with p16+/hrHPV DNA+ tumours was 85% [36], 78% [32], 59% [33], 87% [34] and 66% [35] in the five studies. For all studies pooled, 350 of 459 (76%) patients with ASCC tested p16+/hrHPV DNA+. Fifty-two patients (11%) were negative for both markers. Thirty-one tumours (7%) were p16+/hrHPV DNA-, and 26 (6%) tumours were p16-/hrHPV DNA+.

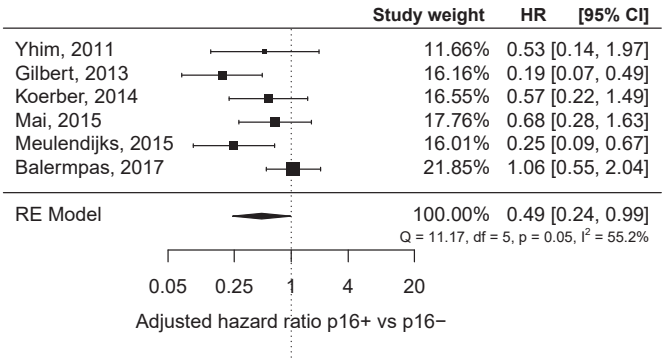

Fig. 3. Forest plot of adjusted hazard ratios (HRs) comparing survival in patients with p16+ versus p16- ASCC, adjusted for age, gender, T-stage and N-stage. CI, confidence interval; RE, random-effects.

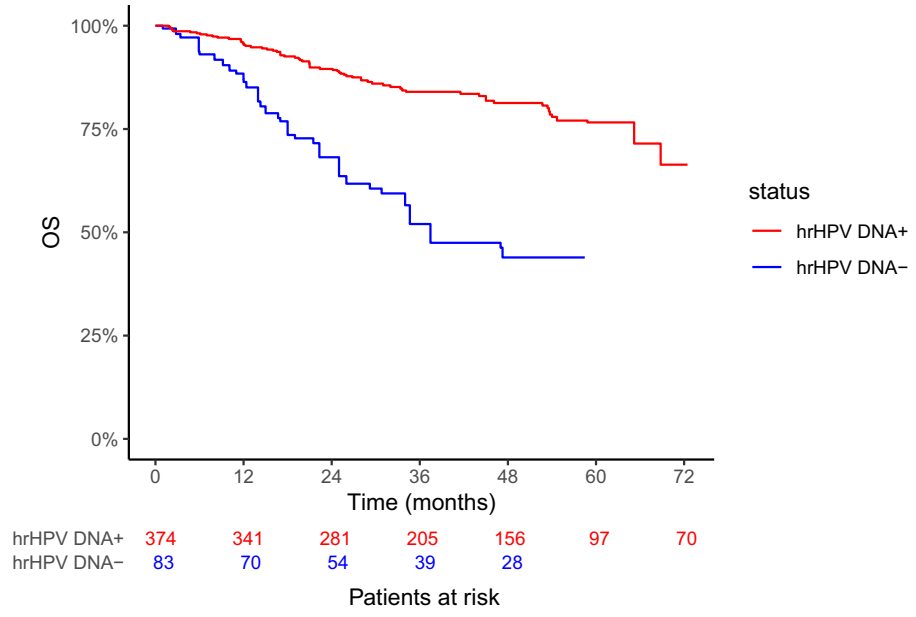

Fig. 4. Pooled overall survival (OS) curves stratified by hrHPV DNA status in patients with anal cancer of the five studies included in the main analysis (for patients with known hrHPV DNA status and outcome information) and weighted by the study's total sample size. The number of patients under observation at risk of dying at the beginning of each year is mentioned under the X-axis. hrHPV, high-risk human papillomavirus.

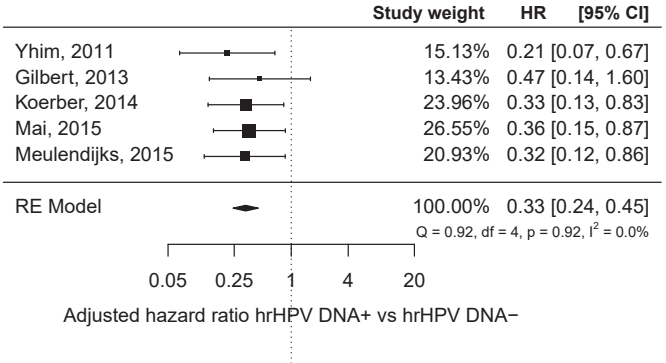

Fig. 5. Forest plot of hazard ratios (HRs) comparing the survival of patients with hrHPV DNA+ ASCC with that of patients with hrHPV DNA- ASCC, adjusted for age, gender, T-stage and N-stage. CI, confidence interval; RE, random-effects; hrHPV, high-risk human papillomavirus.

3.4.1. Survival in patients as per the combined p16 and hrHPV DNA status

The RE model showed that the pooled adjusted hazard was significantly lower for the p16+/hrHPV DNA+ than that for the p16-/hrHPV DNA- status (HR = 0.26 [95% CI, 0.14–0.50]; Fig. 6). The pooled adjusted hazard for the p16-/hrHPV DNA+ group was also significantly lower than that for the p16-/hrHPV DNA- group (HR = 0.52 [95% CI, 0.33–0.83]). No significant difference was observed in the pooled adjusted hazards between patients with a p16+/hrHPV DNA- and a p16-/hrHPV DNA- tumour (p = 0.6498) (Table 4). Patients with double-positive tumours (p16+/hrHPV DNA+) showed superior survival compared with all other groups (Table 4). This relation was statistically significant when comparing double-negative (p16-/hrHPV DNA-) tumours (HR = 3.82 [95% CI, 2.01–7.24]) or p16+/hrHPV DNA- tumours (HR = 3.19 [95% CI, 1.41–7.23]) with this group, but not significant for p16-/hrHPV DNA+ tumours (HR = 2.33 [95% CI, 0.89–6.09]).

The pooled three-year survival rate in patients with p16+/hrHPV DNA+ ASCC was 86% (95% CI 82–90%) compared with 39% (95% CI, 24–54%) in patients with p16-/hrHPV DNA- ASCC. In patients with a discordant status, the three-year survival rate was 55% (95% CI, 35–71%) in patients with p16-/hrHPV DNA+ and 75% (95% CI, 56–86%) in patients with p16+/hrHPV DNA- ASCC (Fig. 7).

4. Discussion

The incidence of anal cancer has been rising worldwide throughout the past decades. In Europe, incidence rates have, on average, increased by 23.7% and 26.6% every five years between 1988 and 2012 in men and women, respectively [5]. In the United States, incidence rates have been increasing annually by 2.7% between 2001 and 2015 [45]. The growing disease burden has coincided with intensive research on the aetiology of anal carcinogenesis, which could support the development of refined treatment opportunities. Various studies identified oncogenic (hr) HPV types as causative agents in the majority of anal cancers, and several authors also reported better clinical outcomes for patients with HPV-associated than for those with HPV-negative anal cancers (reviewed in [30,31]). Given the emerging discussions about the value of therapy de-escalation in patients with HPV-induced anal cancer, the prognostic relevance of HPV-related markers gains particular momentum. In the light of those developments, we sought to determine the prognostic value of HPV in anal cancers by conducting an IPD meta-analysis. This allows the assessment of hrHPV DNA and p16 alone and in combination as well as potential confounding factors within multivariate analyses.

In the pooled cohort of 459 patients derived from five eligible studies, we observed a significantly reduced mortality during the observation period in patients with a combined p16+ and hrHPV DNA+ status in their anal cancers compared with patients who were negative for both markers (pooled adjusted HR = 0.26 [95% CI, 0.14–0.50]). The pooled three-year survival rates in the double-positive and double-negative groups were 86% (95% CI, 82–90%) and 39% (95% CI, 24–54%), respectively. Combined detection of hrHPV DNA and p16 overexpression has been suggested as a reliable and practical approach to identify an aetiological role of HPV in malignant tumours in different sites of the genital and head and neck region [20,23,44]. Assuming aetiological relevance of HPV in anal cancers that are both p16+ and hrHPV DNA+, the pooled prevalence of HPV-induced anal cancers in our meta-analysis was 76%. This proportion reflects an average among five studies (459 patients) with prevalence rates ranging from 59% to 87%. Importantly, our meta-analysis indicates that the average proportion of presumably HPV-induced cancers is situated in the lower range of previous estimations that were based solely on HPV DNA detection (about 70%–95%; [8,46–48]).

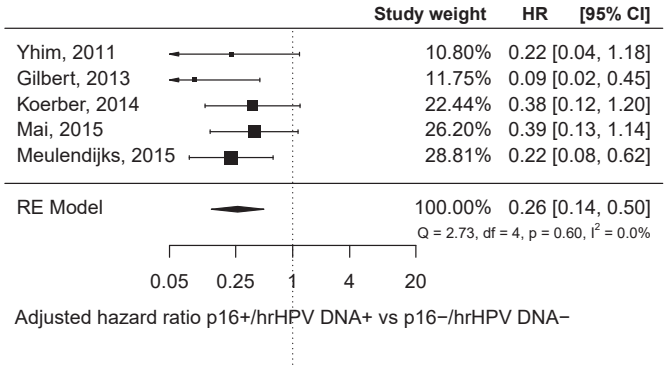

Fig. 6. Forest plot of hazard ratios (HRs) comparing the survival of patients with p16+/hrHPV DNA+ ASCC with patients with p16-/hrHPV DNA- ASCC, adjusted for age, gender, T-stage and N-stage. CI, confidence interval; RE, random-effects; hrHPV, high-risk human papillomavirus; ASCC, anal squamous cell carcinoma.

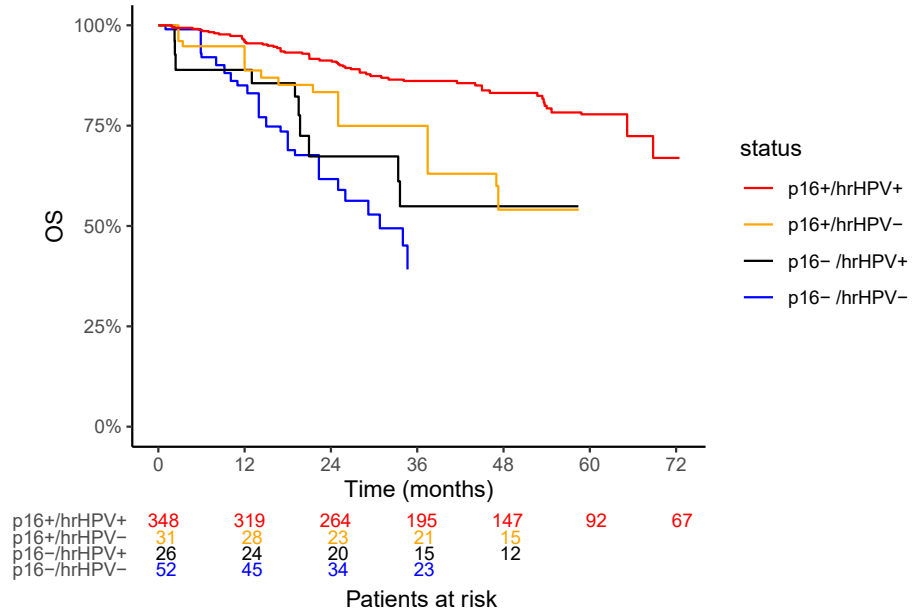

Fig. 7. Pooled overall survival (OS) curves stratified by the combined p16 and hrHPV DNA status in patients with ASCC (for patients with known p16 and hrHPV DNA status and outcome information) and weighted by the study's total sample size. The number of patients under observation at risk of dying at the beginning of each year is mentioned under the X-axis. hrHPV, high-risk human papillomavirus; ASCC, anal squamous cell carcinoma.

Table 4
Pooled hazard ratios (HRs) of p16 and hrHPV DNA status combinations, adjusted for age, gender, T-stage and N-stage for five included studies, using the double-negative (top) and double-positive cases (bottom) as reference.

Index group	Reference group	Adjusted HR	95% CI	p
p16+/hrHPV DNA+	p16-/hrHPV DNA-	0.26	0.14–0.50	0.0044
p16-/hrHPV DNA+		0.52	0.33–0.83	0.0205
p16+/hrHPV DNA-		0.78	0.19–3.21	0.6498
p16-/hrHPV DNA-		1.00	–	–
p16+/hrHPV DNA+	p16+/hrHPV DNA+	1.00	–	–
p16-/hrHPV DNA+		2.33	0.89–6.09	0.0672
p16+/hrHPV DNA-		3.19	1.41–7.23	0.0171
p16-/hrHPV DNA-		3.82	2.01–7.24	0.0044

CI, confidence interval; hrHPV, high-risk human papillomavirus.

The significantly better survival rates of the p16+/hrHPV DNA+ compared with the p16-/hrHPV DNA- patient group imply that HPV aetiology in anal cancers is a major determinant of patient prognosis. Improved prognostication by combined p16 and hrHPV DNA detection compared with single-marker analysis has also been demonstrated in a large meta-analysis on tumours in the head and neck region [25].

It has been suggested that the superior survival rates of patients with HPV-induced, that is p16+ and hrHPV DNA+, cancer could be explained by distinct biological treatment responses irrespective of the anatomical origin of the tumour. This hypothesis is supported by studies reporting a higher sensitivity of HPV-transformed tumour cells than that of HPV-negative cell lines from oropharyngeal cancers towards radio(chemo)therapy [49–51], which also represents a central treatment component for patients with anal cancer. The heterogeneity of reported treatment data among the included studies in our meta-analysis precluded the determination of treatment effects on patient prognosis. However, considering the favourable prognosis of patients with HPV-induced cancers observed in our study and the resulting prospect of treatment de-escalation in these patients, future studies should address the influence of treatment modalities on HPV-induced anal cancers in greater detail.

The determination of possible aetiological factors in the group of p16-/hrHPV DNA- ASCC was not feasible on the basis of the obtained IPD in our meta-analysis. However, considering that patients with these cancers displayed poor survival rates and represented more than 10% of all patients with ASCC in our meta-analysis, it is of high clinical relevance to investigate potential carcinogenic risk factors in this patient group in future studies, thereby exploring whether any preventive measures might be feasible.

Overall, we observed a gradient of increasing mortality among the four different groups (p16+/hrHPV DNA+ < p16-/hrHPV DNA+ < p16+/hrHPV DNA- < p16-/hrHPV DNA-). Several authors have advocated against the use of either p16 or HPV DNA alone as indicators of HPV-induced aetiology in cancers, but recommend their combined use to reliably differentiate HPV-induced from HPV-unrelated tumours [44,52,53].

This caution is on the one side based on indications that tumours with a discordant hrHPV DNA/p16 status might represent biologically and clinically distinct tumour entities that could be accompanied by different mortality rates [25]. On the other side, the discordant status might also result from misclassifications of either p16 or HPV DNA: contamination of tumour specimens with environmental hrHPV DNA during tumour collection or sample processing [54,55,62] or overly sensitive detection methods may, for example, account for a false-positive hrHPV DNA test result and thus classification of the tumour as p16-/hrHPV DNA+ while it is in fact HPV-negative (p16-/hrHPV DNA-). The true HPV status and the associated clinical prognosis in cases with a discordant p16/hrHPV DNA status could thus be masked, resulting in their positioning between the mortality rates for HPV-induced (p16+/hrHPV DNA+) and HPV-negative (p16-/hrHPV DNA-) cases. Considering the small patient numbers in the groups with a discordant p16/hrHPV DNA status in our meta-analysis, we cannot derive firm conclusions about differential survival and relative prognosis between the two groups at this point. We therefore recommend continuing efforts to collect IPD data from more and larger studies to disentangle this question with more power. This appears particularly important considering discussions on treatment de-escalation based on the tumoral HPV status.

Prevalence rates for p16, hrHPV DNA, and combined p16 and hrHPV DNA detection were significantly higher in female than those in male patients with anal cancer in the pooled cohort of our meta-analysis (83% vs. 65%, $p < 0.0001$ for p16+, 89% vs. 71%, $p < 0.0001$ for hrHPV DNA+, and 84% vs. 63%, $p < 0.0001$ for combined p16+/hrHPV DNA+ tumours). Possible explanations for the observed differences could relate to a higher risk of autoinoculation with HPV from genital sites particularly in women with genital HPV-induced precancerous lesions [56,57] as well as to a reduced ability to clear hrHPV infections in women [58]. Our meta-analysis data indicating a higher proportion of HPV-induced ASCC in female than in male patients are in line with epidemiological data demonstrating significantly higher incidence rates of ASCC in women than in men [4].

4.1. Strengths and limitations

To the best of our knowledge, this is the first IPD meta-analysis assessing the prognostic value of HPV-related markers in anal cancers. We could compile a large pooled cohort of 693 patients derived from seven original studies in five different countries, enabling us to control for several confounding factors, such as age, gender, T-stage and N-stage, in our multivariate analyses. Furthermore, the provision of data on detected HPV types in the original studies allowed us to specifically focus on DNA of hrHPV types, refining our studies to biologically meaningful markers that can indicate transforming relevance of HPV in the anal cancers. We could further study the relation between p16/hrHPV DNA and the clinical course over a follow-up period of up to 72 months in the total cohort, thereby providing long-term data on the prognostic significance of those markers.

Our meta-analysis also holds some limitations that may confine generalisability of the observed results. First, the prognostic relevance of p16 in ASCC was of major interest in the study design of this meta-analysis, and the availability of p16 IHC data therefore represented a central inclusion criterion. Consequently, studies reporting hrHPV DNA data but no p16 IHC data were excluded, thereby limiting the number of included studies to assess the prognostic value of hrHPV DNA. Moreover, study sizes are rather small given the rarity of anal cancer. In particular, the groups with a discordant p16 and hrHPV DNA status were small, downgrading the quality of evidence derivable from our meta-analysis on these groups. The methods and cut-off definitions for the detection of hrHPV DNA and p16 differed considerably among the seven included studies (Supplementary Tables 2 and 7), which could explain the large variability of prevalence rates of the markers observed in this meta-analysis. This challenge has also been recognised for HPV-related analyses in other anatomical sites [23,59,60] and might be resolved at least partly by the introduction of consensus cut-offs for the individual test methods in future studies. We performed an *a posteriori* modification of an HPV 16 DNA cut-off definition for one study included in this meta-analysis [38] that more closely resembled the ones used in the other studies. Sensitivity analyses (Supplementary Tables 9 and 10) showed that the prognostic value of hrHPV DNA was not considerably altered when this study was excluded from the pooled evaluation after *a posteriori* modification of the cut-off definition (Fig. 5 and Table 4). This finding corroborates the value of consensus definitions for test interpretation in HPV-related analyses. We therefore highly encourage the conduct of large cohort studies specifically comparing the prognostic value of different definitions of p16- and hrHPV DNA-positivity as a basis for a future consensus definition in ASCC. Finally, information on tumour treatment was not available for all included studies and was largely heterogeneous for the patients with available data, precluding analyses on treatment modality as a potential confounding factor. Another limitation is that certain statistical analyses are

restricted to the follow-up duration of all studies and therefore determined by the shortest study.

5. Conclusion

We demonstrated for the first time the superior prognostic value of combined hrHPV DNA and p16 compared with their individual detection in an IPD meta-analysis on patients with anal cancer. Patients with HPV-unrelated (p16-/hrHPV DNA-) anal cancers showed a poor three-year survival rate of only 39%, whereas this rate was doubled (86%) in patients with HPV-induced (p16+/hrHPV DNA+) tumours. In contrast to previous meta-analyses that were based on extracted aggregated data, we were able to control for confounding variables. In the light of the rising incidence rates of anal cancer and the prospect of differential treatment of affected patients in relation to the tumoral HPV status, our findings invite for more trials aiming to optimise future treatment of patients with anal cancer in accordance with HPV aetiology.

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Author contributions

ESP, MR and TO designed the study. TO reviewed titles, abstracts and full-text articles. ESP cross-checked the full-text articles. LB, AC, DCG, SAK, SM, DM, FR and HYY prepared and provided individual patient data. TO compiled and formatted IPD. JH, MPB, DR, EG and MA wrote the statistical analysis plan. JH, MPB and DR performed the statistical analyses. ESP and TO cross-checked the computed data. ESP, TO, JH and MA created the manuscript draft. MPB, DR, LB, AC, DCG, SAK, SM, DM, FR, HYY, SH, CW, CLR, AU, FV, SKK, MR, EG and MvKD critically revised subsequent drafts. All authors read and approved the submitted version.

Conflict of interest statement

S.A.K. received grants from ViewRay Inc. and honoraria from IBA Dosimetry, outside the submitted work. S.K.K. has previously received speaker fee from Merck and a research grant through her institution from Merck outside the submitted work. D.M. is an employee and shareholder of AstraZeneca Ltd., UK. E-S.P. has received lecture fees by MSD Sharp & Dohme GmbH and Institut für Frauengesundheit (IFG) GmbH. E-S.P. is involved in a research project funded by MSD Sharp & Dohme GmbH outside the submitted work. M.R. is an inventor of patent applications related to therapeutic targeting of $p16^{INK4a}$. M.R. is meanwhile an employee of MSD Sharp & Dohme GmbH, but completed all work associated with this manuscript while full-time employed at Heidelberg University Hospital. F.V. received a grant from the Danish Council for Independent Research outside the submitted work. M.v.K.D. is involved in a research project funded by MSD Sharp & Dohme GmbH outside the submitted work and has received consulting and lecture fees from MSD, Roche and Oncgnostics. All remaining authors have declared no conflicts of interest.

Appendix A. Supplementary data

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