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Women in head and neck cancer: 2021

Edited by
Amanda Psyrri, Panagiota Economopoulou and Shao Hui Huang

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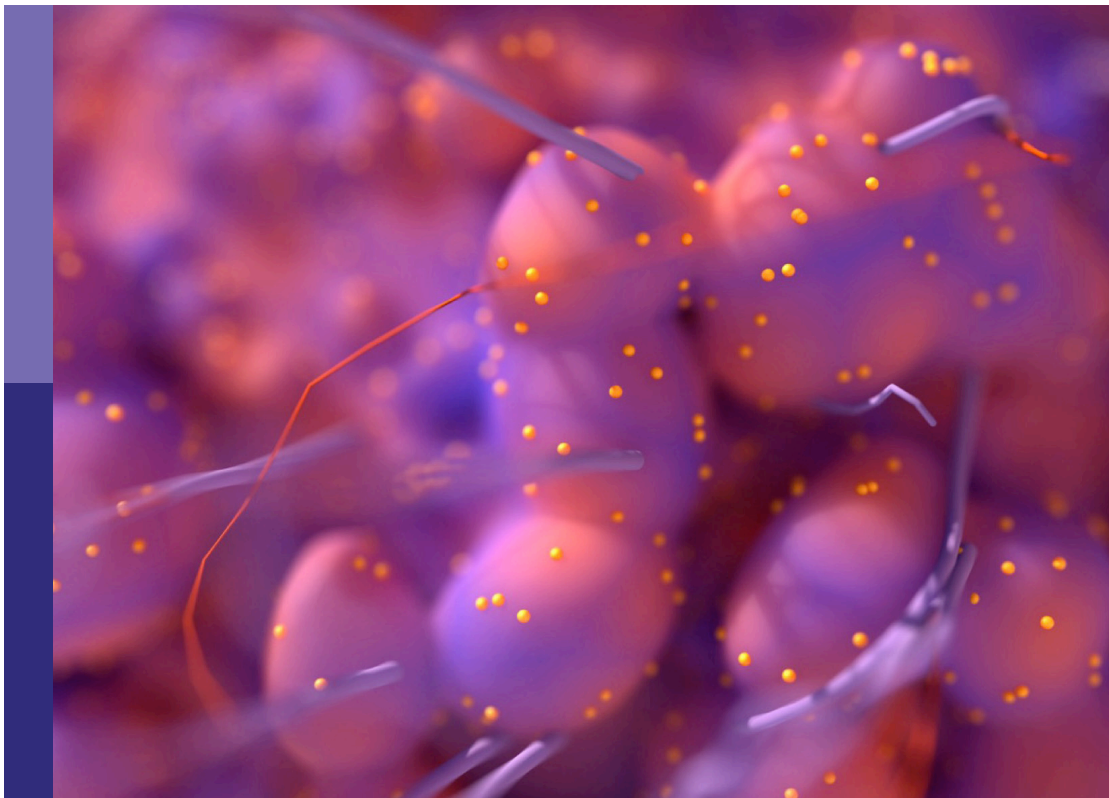

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Women in head and neck cancer: 2021

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Editorial: Women in head and neck cancer 2021

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Editorial on the Research Topic
Women in head and neck cancer 2021

In the Research Topic that accompanies this editorial, seventeen articles were included that cover various topics in head and neck cancer, such as systemic therapy, immunotherapy, novel therapeutic targets, analysis of surgical techniques and their complications, radiomics, prognostic and predictive biomarkers. This special issue showcased important contribution from female colleagues in the head and neck oncology.

As demonstrated in the Keynote 048 phase III trial (1), Programmed Death 1 (PD1) checkpoint inhibitor pembrolizumab, either as monotherapy or in combination with chemotherapy, increased overall survival in recurrent metastatic (R/M) squamous cell carcinoma of the head and neck (HNSCC) for patients whose tumors exhibit high expression of PDligand-1 (PD-L1), as defined by a combined positive score (CPS) ≥ 1. On the contrary, for tumors with PD-L1 CPS<1, the EXTREME regimen was shown to be equivalent to chemo/immunotherapy combination (2). In the current Research Topic, Lubbers et al. presented real-world data from a retrospective analysis that included 124 patients with R/M HNSCC before the approval of immunotherapy as first line therapy. Further supporting the results of the EXTREME trial (3), it was shown that the EXTREME regimen was superior to all other chemotherapy regimens in the 1st line setting. Thus, in patients with PDL1 CPS score <1 or who have major contraindications for immunotherapy administration, EXTREME remains the standard of care. Nevertheless, there is an unmet need for more chemotherapy options for patients with severe comorbidities who are particularly frail and unfit for EXTREME. In this context, Carinato et al. presented retrospective data from 60 patients who were treated with weekly carboplatin, paclitaxel and cetuximab and found comparable survival outcomes with the standard EXTREME regimen.

Despite a remarkable progress in the treatment of head and neck tumors, a minority of patients derive actual benefit from approved therapies, and the only clinically relevant predictive biomarker is PD-L1 CPS score. Current studies focus on the development of clinical and molecular predictive or prognostic biomarkers that could be associated with clinical aggressiveness, response to therapy or clinical outcomes. Arribas et al. showed that low skeletal muscle index (SMI) at baseline, that was calculated *via* SliceOmatic software using

a CT scan with the 3rd lumbar vertebra as a reference point, might have a negative impact on survival in patients receiving immune checkpoint inhibitors. On the other hand, Giunco et al. evaluated surgical specimens from patients with oral cavity squamous cell carcinoma (OSCC) and found that the coexistence of a specific mutation in the TERT promoter (-124 C>T) with a single nucleotide polymorphism (T/T genotype of the rs2853669) is associated with a poor prognosis in patients with early disease.

In early HNSCC, surgery has been shown to confer favorable outcomes depending on the primary site of the tumor. Laryngectomy has been considered the gold standard in advanced disease, either as primary or salvage therapy. However, several complications such as pharyngocutaneous fistula, are relatively common and lead to delays in adjuvant treatments and decline in quality of life. Using a very strict surgical protocol, an Italian group managed to reduce rates of this complication (Crosetti et al.). On the other hand, for very advanced disease involving the skull base that is stratified as T4b, carotid-sparing surgery or surgery including total carotidectomy is a therapeutic option that can lead to cure, albeit with serious sequelae such as carotid blowout. As meticulously discussed by Orlandi et al., every patient with carotid artery encasement is a unique case that requires management by multidisciplinary team including surgeons, radiotherapists, medical oncologists and interventional radiologists.

On the other hand, a transcriptionally active infection with Human Papilloma Virus (HPV), mainly HPV16, is a well-characterized contributing factor that is etiologically linked to a biologically distinct subgroup of oropharyngeal tumors with different clinical presentation and improved prognosis. Furthermore, patients with HPV-driven cancers do not commonly develop second primary tumors, which typically arise in the mucosa of the upper aerodigestive track following the accumulation of tobacco and alcohol-induced genetic alterations. In this context, Guarda et al. sought to assess the possibility of the development of synchronous and metachronous HPV-related oropharyngeal tumors by estimating the prevalence of a transcriptionally active HPV infection in the normal appearing mucosa next to and distant from the tumor. Interestingly, HPV was identified but not transcriptionally active in the cancer-free oropharyngeal tissue, indicating that the phenomenon of a field-cancerization prompted by HPV may not relevant in HPV-related oropharyngeal cancer.

Moreover, salivary gland carcinomas are relatively rare tumors that account for less than 5% of head and neck malignancies. A correction of an original review article discussing novel targets for advanced disease provides two precisely structured algorithms for adenoid-cystic and non-adenoid cystic carcinomas (Di Villeneuve et al.). Among all histological subtypes, mucoepidermoid carcinoma (MEC) is the most commonly encountered. Genetic studies may unravel biomarkers that can facilitate diagnosis and prognosis of MEC. Naakka et al. performed a miRNA and array-based gene expression analyses in 35 fresh frozen MEC samples and six normal salivary gland tissues and found that increased expression of mir-205 and mir-22 were associated with worse prognosis (). Interestingly, inhibition of these miRNAs in a MEC cell line resulted in reduced viability and invasion. Last, Piludu et al. highlighted the importance of MRI-based radiomics to enable differentiation of parotid lesions.

In conclusion, the present Research Topic has gathered several influential articles that sought to illustrate current knowledge regarding novel therapies and clinical/molecular biomarkers for head and neck carcinomas.

Author contributions

All authors listed have made a substantial contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Delayed Response After Confirmed Progression (DR) and Other Unique Immunotherapy-Related Treatment Concepts in Cutaneous Squamous Cell Carcinoma

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Non-melanoma skin cancers are one of the most common cancers diagnosed worldwide, with the highest incidence in Australia and New Zealand. Systemic treatment of locally advanced and metastatic cutaneous squamous cell carcinomas has been revolutionized by immune checkpoint inhibition with PD-1 blockade. We highlight treatment issues distinct to the management of the disease including expansion of the traditional concept of pseudoprogression and describe delayed responses after immune-specific response criteria confirmed progressive disease with and without clinical deterioration. We term this phenomenon "delayed response after confirmed progression (DR)". We also discuss the common development of second primary tumors, heterogeneous disease responses, and expanding clinical boundaries for immunotherapy use.

Keywords: immunotherapy, PD-1 inhibition, pseudoprogression, cutaneous squamous carcinoma, second primary tumors (SPTs)

INTRODUCTION

Non-melanoma skin cancers (NMSC) predominantly basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (CSCC) are the most frequently diagnosed cancer in North America and Australia/New Zealand (1). Although most are resectable, the morbidity related to disease is significant and accounts for the most common cancer-related cause for hospitalization in Australia exacerbated by the multiplicity of skin cancer excisions (2–4). Approximately 5% of CSCC recur or metastasize leading to death or management associated with significant morbidity due to disease occurrence on sun-exposed areas such as the face, head and neck (5, 6).

In 2018, the first report of the efficacy of cemiplimab, a PD-1 inhibitor, was published for the treatment of patients with locally advanced or metastatic CSCC who were not candidates for curative surgery or radiation. The objective response rate (ORR) to therapy was 47% (range: 34-61),

leading to Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval and a paradigm shift in the management of these tumors (7, 8). Updated data indicates that the median duration of response and median overall survival (OS) have not been reached, with estimated 24 month-OS being 73.3% (95% CI: 66.1-79.2) (9). The KEYNOTE-629 study showed that pembrolizumab is also efficacious (ORR 34%; 95% CI: 25- 44) (10), which also led to FDA approval. First line pembrolizumab in the CARSKIN trial for locally advanced or metastatic CSCC (including radiotherapy naive patients, n=20/57) achieved a week 15 ORR of 41% (95% CI: 26-58%) (11). Therefore, the use of PD-1 blockade for the treatment of advanced CSCC represents a major breakthrough in the management of these common epithelial cancers.

We report our immunotherapy management experiences unique to CSCC that challenge and expand current clinical concepts in practice.

EXPANDING ON THE CONCEPT OF PSEUDOPROGRESSION – "DELAYED RESPONSES AFTER CONFIRMED PROGRESSION (DR)" AND RESPONSE AFTER CLINICAL DETERIORATION

The Response Evaluation Criteria in Solid Tumors (RECIST) criteria is a validated measure for the standardized evaluation of cancer therapies, determined by the assessment of the change of tumor burden with treatment (12). There are notable limitations of the RECIST guidelines in patients treated with immunotherapy, given that "pseudoprogression" can occur with an increase in tumor size due to inflammatory cell infiltrates followed by tumor reduction (13), and improved OS can occur without RECIST defined reduction in tumor measurements (14–16). Thus, RECIST criteria have been modified to include immune-related response criteria (irRC), immune-related response evaluation criteria in solid tumors (irRECIST), modified RECIST1.1 for immunotherapy (iRECIST), and immune-modified RECIST (imRECIST) (17–20).

Pseudoprogression occurs in under 10% of all cancers treated with immunotherapy, with an incidence in head and neck cancer of approximately 1% (21–24). To address pseudoprogression, the irRC, irRECIST and iRECIST require the use of confirmatory imaging at least 4 weeks after initial progression is documented (we will refer to this collectively as iCPD, immune confirmed disease progression as per iRECIST), a minimum size increase of >5x5mm^2 or 10mm, an increase in the sum of tumor measurements from the nadir of 20-25%, and incorporation of new lesions in the sum of tumor dimension measurements before confirmation of progressive disease (PD) (17–19). The imRECIST criteria is similar, but permits the best response assessment to occur after observation of progressive disease, which avoids underestimation of survival rates (20). All criteria recommend that treatment beyond progression should only occur if a patient's performance status and disease-related symptomatology are stable.

We describe two cases to redefine our understanding of pseudoprogression, with delayed disease response observed after iCPD and after observation of clinical deterioration. We introduce new terminology to capture the phenomenon as "delayed response after confirmed progression (DR)". We also discuss a case of tumor response observed in a patient with clinical deterioration, with treatment beyond progression.

Case One

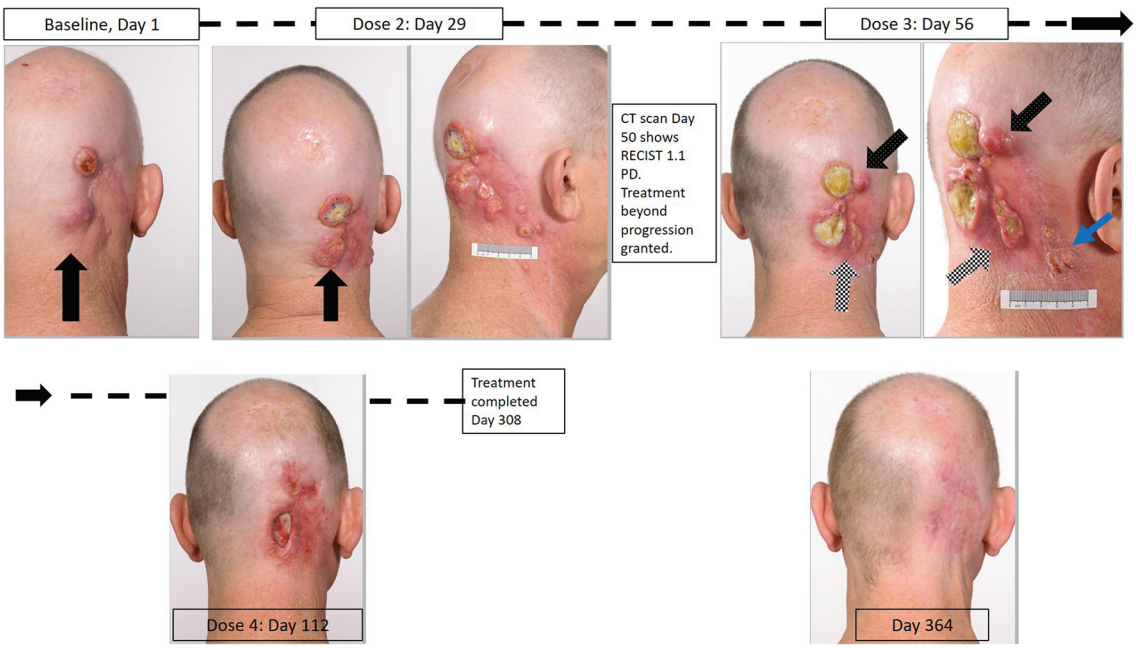
This case illustrates the observation of DR. Patient One was diagnosed with a T2N0M0 occipital scalp/vertex CSCC excised with clear margins in September 2017. In July 2018, a 35mm in-transit recurrence was resected with an involved margin. Re-excision and ipsilateral neck dissection demonstrated a 2.5mm residual CSCC 1.2mm from the deep margin, and 1/41 nodes involved with extranodal extension to the surgical margin. Adjuvant radiotherapy of 66 Gy/33# was completed in October 2018. Within three weeks, biopsy of multiple new in-field in-transit CSCC and an out-of-field intramuscular lesion confirmed recurrent disease. Following multidisciplinary meeting (MDM) discussion, he was referred for consideration of immunotherapy for his recurrent CSCC.

The patient was enrolled on the NCT02760498 to receive cemiplimab 400mg Q4W, with RECIST 1.1 measurements determined by radiological assessments as per protocol. The progression of lesions documented by photography, and radiological assessments are summarized in **Figures 1A, B**. Four weeks after initial dose, Patient One reported worsening disease-related pain. The largest CSCC lesion had increased by 10mm with new ulceration (black arrow), other baseline lesions had also increased in size with ulceration, while at least three new lesions greater than 10mm had developed with multiple other smaller lesions visible. This corresponded to unconfirmed progressive disease (iUPD). Four weeks later prior to his third dose, ongoing progression was noted with further ulceration and coalescence of lesions (white checked arrow), and increasing size of new nodules by more than 10mm (black spotted arrow) confirming iCPD. At that time point, RECIST 1.1 assessment by imaging confirming PD (**Figure 1B**). Approval for treatment beyond progression was granted. By the next visit, all lesions had improved clinically with a reduction in size and improvement in pain. The patient completed 12 months of therapy with a complete clinical response. To date, the patient remains in clinical and radiological remission.

Case Two

This case illustrates DR. Patient Two had a long history of multiple NMSC lesions being excised from the head, neck, and chest, including a Merkel cell carcinoma (MCC). Patient Two had a CSCC lesion from the left clavicular area that required multiple re-excisions over a six-month period before clear margins were achieved in January 2018. In May 2018, a left infraclavicular chest recurrent CSCC was excised that involved

A



B

Target Lesion Site (in mm)		Baseline RECIST	Clinic review Dose 2 Day 28	CT Day 50	Clinic review Dose 3 Day 56	CT Day 108	CT Day 167	CT Day 335	CT Day 386 Follow up 2 months	CT Day 442 Follow up 4 months
A	Right paraoccipital subcutaneous lesion	31x21	Progression, ulceration, increased pain	37x29	Progression, ulceration, increased pain	29 x 20	25x18	16x6	Too small to measure default 5mm	Too small to measure default 5mm
B	Right neck intramuscular deposit	14 x10	Confluent progression with other lesions	17 x 15	Progression, ulcerated, increased pain	12 x 12	0	0	0	0
Total (Target) mm		**45**		**54**		**41**	**25**	**16**	5	5
% change from baseline **% change from re-baseline after iUPD**		NA		+20		-9 -24	-44 -53	-64 -70	-89 -91	-89 -91
Non-Target Lesion Site			**Present (P) or Absent (A)**							
1 Dermal deposits right neck		Present		Present		Present	Absent	Absent	Absent	Absent
New lesions			At least 3x10mm	Yes, multiple new scalp and neck	Increased further by >20mm but no new lesions	No	No	No	No	No
Overall Response RECIST 1.1			**PD**	**PD**	**PD**	**PD**	**PD**	**PD**	**PD**	**PD**
Including clinical assessments **iRECIST** **irRECIST** **imRECIST**			**iUPD** **iUPD** **iUPD**	**iCPD** **iCPD** **iCPD**	**iCPD** **iCPD** **iCPD**	**iCPD** **iCPD** **iSD**	**iCPD** **iCPD** **iPR**	**iCPD** **iCPD** **iPR**	**iCPD** **iCPD** **iPR**	**iCPD** **iCPD** **iPR**

FIGURE 1 | Continued

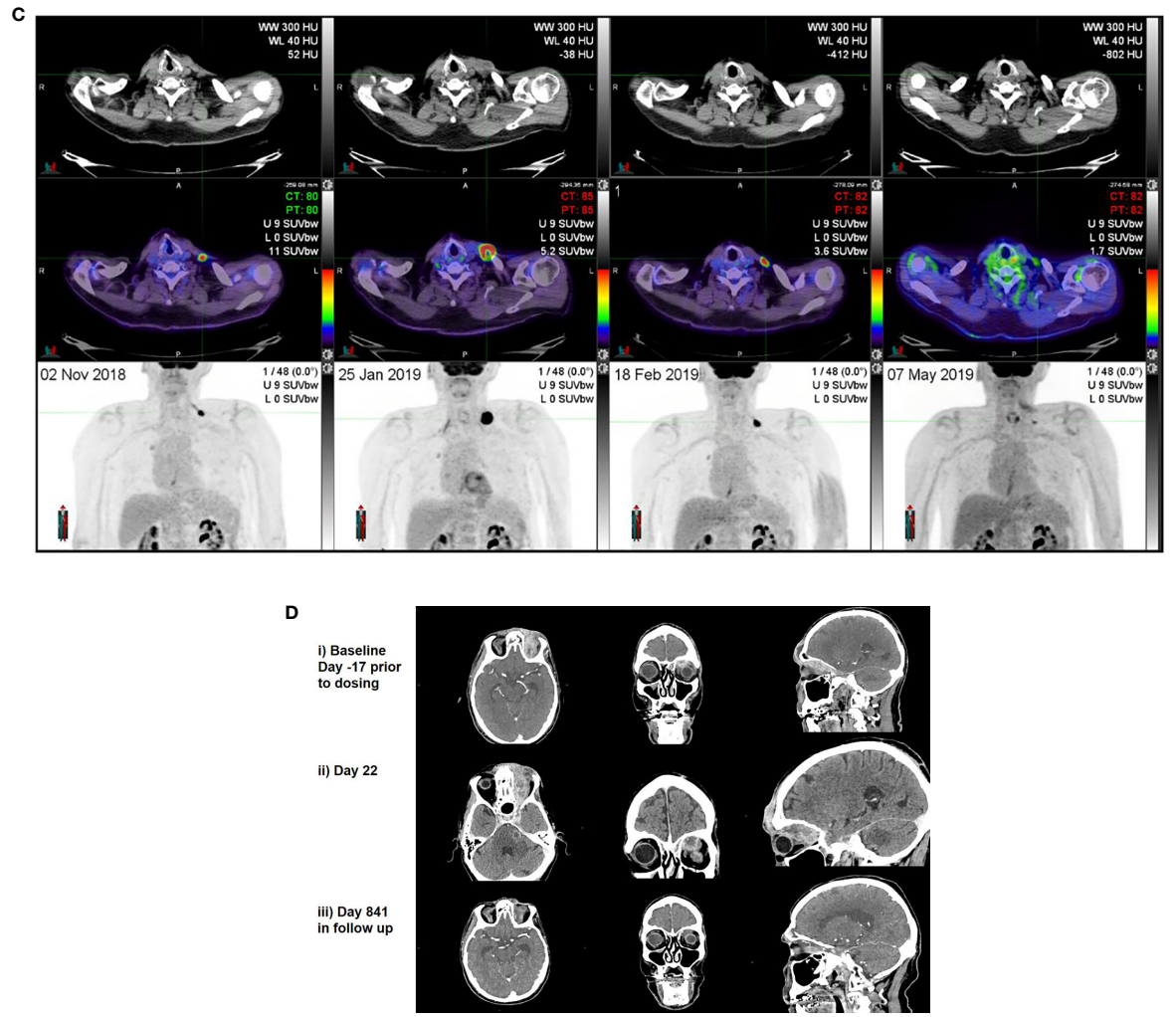


FIGURE 1 | Correlative clinical photographs, tumor response measurements according to time, and fluorodeoxyglucose - positron emission tomography (FDG-PET) and CT scan images of discussed patient cases. **(A, B)** Patient One's photographic images demonstrating iUPD and iCPD, with accompanying tumor response measurements according to RECIST 1.1 and immune based criteria at key time points. **(C)** Patient Two's FDG-PET images over time with low dose axial CT, fused axial CT, and maximal intensity projection (MIP) images in each column from 02/NOV/2018, 25/JAN/2019, 18/FEB/2019, and 07/MAY/2019, demonstrating evidence of progression and regression with ongoing immunotherapy. **(D)** Patient Three's representative images of disease at baseline, at progression and with response. Images i) Baseline (Day-17 prior to dosing) Post contrast CT: (axial, coronal, sagittal) demonstrates enhancing soft tissue extending from the cutaneous left forehead along the roof of the left orbit in the distribution of V1, involving the cavernous sinus, Meckel's cave, nerve root entry zone of the left trigeminal nerve and likely involvement of the trigeminal nuclei with soft tissue at the left pons. ii) Day 22 Post contrast CT: (axial, coronal, sagittal) demonstrates increasing volume of enhancing soft tissue extending from the cutaneous left forehead along the roof of the left orbit in the distribution of V1, involving the cavernous sinus, Meckel's cave, nerve root entry zone of the left trigeminal nerve and involvement of the trigeminal nuclei with soft tissue at the left pons. iii) Day 841 (in follow up) Post contrast CT: (axial, coronal, sagittal) demonstrates small volume residual non-enhancing soft tissue extending from the cutaneous left forehead along the roof of the left orbit. The remaining soft tissue has resolved on CT.

the clavicular head of the pectoralis major muscle and pathologically measured 32mm with a deep margin of 0.7mm and evidence of perineural invasion. Re-excision demonstrated no residual disease but another 0.3mm central chest poorly differentiated carcinoma of uncertain origin was concurrently excised with clear margins. Adjuvant radiotherapy of 60Gy/30# was completed on August 2018. Two months later, a biopsy of a nodule over the medial aspect of the left clavicle confirmed recurrent CSCC. Magnetic resonance imaging (MRI) and computer tomography (CT) scans were unable to define the lesion, but fluorodeoxyglucose positron emission tomography (FDG-PET) scan identified an ~13mm left supraclavicular lesion (**Figure 1C**). After MDM discussion, Patient Two was referred for consideration of immunotherapy. Given his history of MCC, he was ineligible for trial participation and self-funded pembrolizumab 200mg Q3W which commenced on 28/NOV/2018. At review prior to the second dose (19/DEC/2018), his lesion had increased by more than 10mm to ~40x30mm with

purpuric discoloration of the intact skin consistent with iUPD. By review prior to the third dose (08/JAN/2019), the lesion had fungated through the skin. A restaging FDG-PET scan (25/JAN/2019) performed after administration of three doses of pembrolizumab demonstrated marked interval progression with increase in size of disease to 33mm confirming iCPD (+254%). On 29/JAN/2019, iCPD was confirmed clinically and the fourth dose of immunotherapy was abandoned and surgical salvage was planned. Two weeks later prior to surgery, the patient reported clinical improvement and repeat imaging performed (18/FEB/2019) demonstrated reduction in tumor size to 15mm (-55% from previous). Pembrolizumab was resumed and by the subsequent visit, he had obtained a complete clinical response. Approximately 12 months of treatment was completed on 02/JAN/2020 and the patient remains in complete metabolic, radiological and clinical response to date.

Case Three

This case highlights that clinical deterioration may not militate against disease response with the use of immunotherapy for the treatment of CSCC. Patient Three had a long history of multiple NMSC and at age 81 years was diagnosed with a T4N0M0 left supraorbital/intraorbital CSCC with perineural disease that involved all branches of the left trigeminal nerve to the cisternal portion. Despite 54Gy in 27 fractions of radiotherapy completed on December 2017, MRI scan in February 2018 revealed disease progression with increasing tumor anterior to the left frontal bone, new marrow infiltration of the floor of the left cranial fossa with nodular dural enhancement and no response in the pre-existing orbital disease (**Figure 1D**). After MDM discussion, he was referred for consideration of immunotherapy. He consented to trial participation in the NCT02760498 (cemiplimab 3 mg/kg Q2W). At Day 1 review, his ECOG performance status was assessed as 1, and examination demonstrated a 25mm left supraorbital mass, complete left eyelid proptosis, complete opthalmoplegia of his left eye, decreased sensation in the trigeminal nerve distribution with patient report of neuropathic pain. At Day 15 his pain had improved but he reported falls at home on the background of worsening balance, resulting in local trauma to the forehead. Clinical examination demonstrated no obvious abnormalities compared to baseline, however ECOG performance status was assessed as 2. Patient Three was referred for allied health management and received his second dose of cemiplimab. Two days later, he was admitted to hospital following a fall with a head strike with loss of consciousness, and reported nausea. CT brain (Day 21) identified disease progression (+44%, iUPD) with enhancement of the left trigeminal nerve disease into the cerebellar peduncle and left-sided pons with surrounding edema. The patient was discharged mobilizing on a wheeled frame the following day. At Day 29 review, the patient reported a significant improvement in his neuropathic pain, the supraorbital mass was clinically smaller but his ECOG performance status was assessed as 2. Restaging CT scans performed (Day 50) identified ongoing disease progression with an increase in size in all target lesions (+42% from baseline, RECIST 1.1 PD, iUPD). MRI brain demonstrated progression with increased diffuse skin thickening, increased enhancement of the orbital and periorbital disease, new destruction of the bones associated with the left frontal and ethmoid sinus and left orbit, soft tissue intracranial extension into the left anterior and middle cranial fossa, with increased trigeminal perineural invasion into the left brainstem. In clinic on Day 57, he reported a complete absence of trigeminal nerve pain and a reduction of the left supraorbital mass to 15mm was noted. Treatment beyond progression was approved. By Day 104, imaging demonstrated ongoing RECIST PD (+28%, iUPD) with subsequent review confirming regression of the exophytic component of the supraorbital CSCC and exposed bone. Patient Three went on to complete 659 days on therapy after experiencing a number of immune-related (≤Grade 2) and other medical adverse events (not treatment-related). His most recent imaging of his target lesions (**Figure 1D**) demonstrated complete resolution of the pons lesion, stable orbital lesion and "not measurable" ulcerated left scalp lesion.

Two of the patient cases presented introduce a new phenomenon we have defined as a "delayed response after confirmed progression (DR)". That is, the observation of a clinical and radiological response after iCPD. In both cases, clinical deterioration was observed early in treatment but after treatment beyond progression, durable clinical and radiological improvement was obtained. In one case, change of tumor evolution with the cessation of new lesions developing may have been the only indication to herald DR. Review of our institutional experience of patients treated with cemiplimab on trial (up to 15/SEP/2020) in the advanced setting who have received more than one dose, demonstrates an estimated incidence of 2/39 (5%) of DR without any cases of "traditional pseudoprogression". We have observed DR occurring late in treatment courses and "traditional pseudoprogression" for non-trial patients. We also raise the concept that clinical deterioration may not militate against tumor response. In distinction to the three cases discussed where all patients experienced disease-related deterioration, the KEYNOTE-629 study which used RECIST 1.1 criteria for response evaluation reported that 29 *clinically stable* patients received treatment beyond progression (10). Of these, 12 patients continue on therapy and eight patients have developed responses (1 complete response, 7 partial responses according to irRECIST; FIG S4 (10)).

Therefore, our observation of DR and scenarios discussed further below, caution against a nihilistic approach to CSCC patient management when using immunotherapy. These cases highlight the important consideration of the timing and method of disease response assessments and the limitations of most criteria to capture pseudoprogression/DR, response rates and best overall response (BOR) assessments as part of clinical trial reporting. Use of the RECIST 1.1 framework will have not accurately reflected BOR for these patients, with the known limitation of most frameworks to act as a surrogate for progression-free and overall survival (25). Although the timing

of imaging assessments are necessarily at a time point to permit sufficient receipt of therapy to assess response, these scans may not contemporaneously reflect disease evolution and response. Further, data from trials that utilize immunotherapy in the neoadjuvant setting prior to definitive surgery suggests that complete pathological responses may be seen only as partial response on imaging (26–29). This highlights the clinical need for research into the predictive and prognostic role of imaging techniques in patients treated with immunotherapy for CSCC and the need to identify relevant molecular liquid biopsy biomarkers for disease surveillance (30–32).

SECOND PRIMARY TUMORS ON IMMUNOTHERAPY

Multiplicity is common for patients with NMSC, with ~74% of all skin cancers being excised from patients with multiple NMSC lesions particularly involving the head and neck region (2, 3). We have observed that patients on immunotherapy can develop both CSCC and BCC as second primary tumors (SPT) despite responsive disease elsewhere, likely due to field cancerization (33). The mechanism by which SPT escape treatment control have not yet been elucidated and are likely to illuminate molecular mechanisms behind immune escape.

From a clinical management perspective, it is critical that SPT development is not mistaken as treatment failure given that these lesions can resolve with ongoing therapy or can be managed with local therapy. As a general principle, the SPT should be observed for a period whilst continuing immunotherapy and if regression or stability does not occur then local therapy can be pursued with a view of continuing systemic therapy for control of the other immunotherapy-responsive disease. On retrospective review of our patients treated with cemiplimab on trial, of eight patients who developed biopsy proven SPT resistant to immunotherapy, seven patients with CSCC and six with BCC required formal excision and/or radiotherapy for local management with all but one patient having more than one lesion. We have not identified any situation for which the new SPT has resulted in metastatic disease or has heralded the development of disease progression in existing lesions. Molecular profiling of these SPTs that develop on immunotherapy is important to define disease heterogeneity and to identify the likely numerous mechanisms of immune escape.

HETEROGENEOUS RESPONSES ON IMMUNOTHERAPY

Discordant immunotherapy responses can be observed between existing lesions, where concurrent local therapy for immunotherapy-resistant lesions may be warranted to secure control. Given the common multiplicity of NMSCs occurring in the same patient (2), awareness of response heterogeneity is key to avoid inappropriate early cessation of immunotherapy.

Case Four

This case demonstrates heterogeneous responses of two baseline lesions to immunotherapy. Patient Four was a 70 year old man with a multiply recurrent CSCC of his left forearm which had required six re-excisions. The largest resected recurrence was a 70x50mm spindled/poorly differentiated CSCC, up to 8mm in depth, with lymphovascular and perineural invasion. Adjuvant 56Gy/28# of radiotherapy was completed in September 2018, with a truncated course due to toxicity. In May 2019, Patient Four developed a painful locally recurrent CSCC of his left elbow with bone on view that measured 41x32mm on MRI scan, and a small right cheek lesion suspected to be a separate primary CSCC. Following MDM discussion, surgery for the cheek lesion was planned prior to immunotherapy for the elbow lesion. Wide local excision of the cheek lesion on the 26/JUN/2019 revealed three areas containing moderately differentiated CSCC spanning over 26mm, 32mm and 40mm with a transected deep margin, multiple CSCC tissue deposits of 2mm to 8mm, with perineural invasion abutting the margin. Re-excision was performed on 03/JUL/2019 revealing multiple foci of moderately differentiated CSCC deposits with perineural invasion, vascular tumor emboli, and tumor 0.2mm from a margin. Patient Four consented to participation the NCT02760498. At baseline, the exophytic left elbow lesion clinically measured 70x35mm. Prior to the third dose (Day 57), a near complete clinical response of the left elbow CSCC was observed with a residual superficial ulcer measuring 10mm accompanied by an improvement in pain. MRI scan confirmed the lesion had reduced to 32x13mm. A month later (Day 87) a right cheek nodule recurred and given its persistence, resection was performed on Day 106 demonstrating an 11mm CSCC with ≤1mm margins. Ongoing immunotherapy secured a complete clinical response for the left elbow lesion by Day 141 with imaging showing no identifiable tumor (nominal RECIST measurement of 5mm). However, three further excisions (Day 147, Day 188, Day 218) were required to manage the cheek lesion which recurred twice during adjuvant radiotherapy planning. Palliative radiotherapy of 36Gy in 6# to the cheek lesion was completed on Day 283. Most recent imaging demonstrated a complete metabolic response (Day 335) and ongoing radiological PR (nominal 5mm, Day 447) of the left elbow disease. However, since Day 394 clinical recurrence of the right cheek nodule has been progressive. Due to the absence of local therapies available to effectively treat the recurrent right cheek CSCC, Patient Four decided to cease further immunotherapy to pursue best supportive care.

Tumoral heterogeneity leading to discordant treatment response is a known therapeutic hurdle that can contribute to disease progression with the development of clonal resistance (34–38). In the era of immunotherapy which can secure durable disease control, understanding the contribution of local therapy towards overall disease control and survival is crucial but poorly understood. It has been observed in patients with melanoma treated with immunotherapy who received local therapy to progressive lesions in order to achieve no evidence of disease, that those who had local therapy to new lesions had poorer

survival compared to those who had local therapy to progressive pre-existing metastases (PFS 6% vs 70%, p=0.001) (39). Consideration of the anatomical site of oligoclonal resistant disease will have clinical (e.g. brain versus lung) and therapeutic implications (e.g. surgery versus radiotherapy). Patient Four's case demonstrates that discordant immunotherapy responses can be observed between lesions, where concurrent local therapy may be warranted in an attempt to secure control of immunotherapy-resistant lesions.

EXPANDING CLINICAL BOUNDARIES - ACTIVITY IN ADVANCED, FUNGATING, DISFIGURING DISEASE AND GOOD TOLERANCE DESPITE MULTIPLE COMORBIDITIES

It is essential to consider patient factors, treatment morbidity and goals of management in oncological care. Comorbidities and

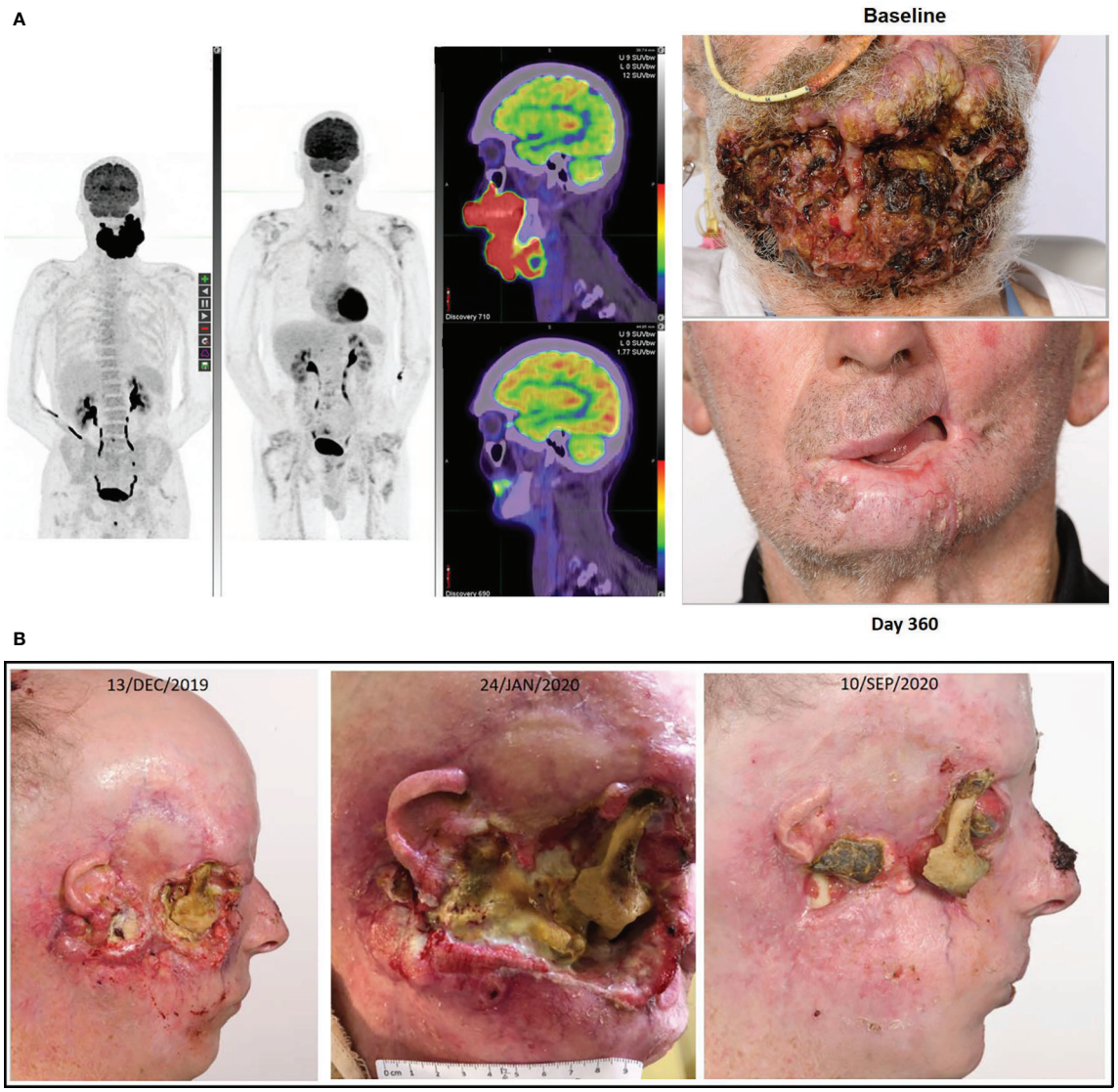


FIGURE 2 | **(A)** This figure illustrates the FDG-PET matched MIP and fused axial images with photographs at baseline and after receipt of cemiplimab of a 75-year-old male who had declined investigation and treatment of CSCC originating from his chin. After 18 months of pursuing alternative treatment, he accepted immunotherapy when the disease had become so advanced it mechanically impacted his ability to eat. He consented to participation in the NCT02760498, and after receipt of two doses clinical regression of the lesion was noted. His disease remains in complete remission after two years of therapy completed more than 12 months ago. **(B)** This figure illustrates re-epithelialization occurring during the receipt of compassionate access cemiplimab in a 45-year-old patient with more than a 20 year history of multiple NMSC including synchronous CSCC, BCC and MCC. The patient ceased vismodegib to commence cemiplimab on 13/DEC/2019 but required recommencement of vismodegib on 19/APR/2020 due to recurrence of multiple BCC lesions. He remains on dual therapy given the symptomatic improvement achieved with good pain control and resolution of right cheek CSCC-related trismus.

ECOG performance status guide management to optimize patient outcomes that may focus on quality of life or may be driven to obtain survival benefits or both. It is well recognized that a large disparity exists between trial patients versus "real-world" patients, who often are older, of minority groups, and with comorbidities that may interfere with assessment of therapeutic efficacy or toxicity (40) (41, 42). In the context of the functionally and cosmetically sensitive anatomical region of the head and neck, it is crucial to define that immunotherapy is generally tolerable and associated with improved quality of life (22, 43, 44). In CSCC, Maubec et al. have reported improved health-related quality of life for patients with immunotherapy-responsive disease (11), and use of PD-1 blockade has been demonstrated to be tolerable with side effects reported similar to other checkpoint inhibitors and with the ability to secure durable disease control (7, 9, 45). Noteworthy in the trial reports, is the median age of patients being 71-80 years with the oldest patients being 99 years old (7, 10, 11).

Our institutional experience in the trial and "real-world" setting is that checkpoint inhibitor therapy is exceptionally well tolerated by patients with CSCC. Generally, few treatment contraindications exist and few comorbidities raise concern including extreme age, dialysis, other synchronous malignancies requiring treatment, and poor ECOG performance status. This is a paradigm change in our approach to patients with CSCC and is paramount given the dramatic response rates achieved by therapy, providing symptomatic and durable control. This is in stark contradistinction to our approach with mucosal head and neck cancer patients. As illustrated in **Figure 2**, there are not many clinical situations in which disease is considered "too advanced" for immunotherapy. That is, the stage of disease, location of disease, and extent of disease does not militate against response to immunotherapy. Anecdotally, the extent of re-epithelialization following response can be impressive creating complexity around the timing of reconstructive surgery if pursued.

CONCLUSION AND FUTURE DIRECTIONS

Use of immunotherapy has revolutionized the care of patients with advanced CSCC, leading to a paradigm shift in the selection of patients for treatment with the expectation of response and durable control even in the advanced recurrent or metastatic setting. We have focused on describing unique clinical concepts related to the treatment of CSCC with immunotherapy including the phenomenon of delayed response after confirmed progression (DR), observation of tumor responses despite clinical deterioration in iCPD, and the need to consider flexible treatment approaches in patients with multiple NMSC.

An improved understanding of CSCC will undoubtedly enhance patient selection for therapy as ongoing clinical research efforts investigate the role of immunotherapy in the adjuvant and neoadjuvant settings. Few cancer registries collect data on CSCC or advanced CSCC, limiting our understanding of the spectrum of disease, burden of need, morbidity and costs related to treatment. The therapeutic advances necessitate rapid development of real-time methods to assess tumor response (e.g. liquid biopsy, imaging or combination approaches) that are more informative than current imaging modalities and response criteria. Translational research will be crucial to molecularly define the clinical spectrum of CSCC (46, 47), and identify reliable predictive and prognostic markers to therapy, including mechanisms of immune evasion. Specifically, comprehensive profiling of immunotherapy exposed tumors, including single cell sequencing approaches, will be important to further clarify inter-tumoral, intra-tumoral and tumor microenvironment molecular processes that underpin the described clinical concepts (48, 49).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peter MacCallum Cancer Centre Ethics Committee. Written consent has been provided by the patient with reidentifiable images.

AUTHOR CONTRIBUTIONS

Concept and design – AL, DR. All authors contributed to the article and approved the submitted version.

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MRI-Based Radiomics to Differentiate between Benign and Malignant Parotid Tumors With External Validation

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Background: The differentiation between benign and malignant parotid lesions is crucial to defining the treatment plan, which highly depends on the tumor histology. We aimed to evaluate the role of MRI-based radiomics using both T2-weighted (T2-w) images and Apparent Diffusion Coefficient (ADC) maps in the differentiation of parotid lesions, in order to develop predictive models with an external validation cohort.

Materials and Methods: A sample of 69 untreated parotid lesions was evaluated retrospectively, including 37 benign (of which 13 were Warthin's tumors) and 32 malignant tumors. The patient population was divided into three groups: benign lesions (24 cases), Warthin's lesions (13 cases), and malignant lesions (32 cases), which were compared in pairs. First- and second-order features were derived for each lesion. Margins and contrast enhancement patterns (CE) were qualitatively assessed. The model with the final feature set was achieved using the support vector machine binary classification algorithm.

Results: Models for discriminating between Warthin's and malignant tumors, benign and Warthin's tumors and benign and malignant tumors had an accuracy of 86.7%, 91.9% and 80.4%, respectively. After the feature selection process, four parameters for each model were used, including histogram-based features from ADC and T2-w images, shape-based features and types of margins and/or CE. Comparable accuracies were obtained after validation with the external cohort.

Conclusions: Radiomic analysis of ADC, T2-w images, and qualitative scores evaluating margins and CE allowed us to obtain good to excellent diagnostic accuracies in differentiating parotid lesions, which were confirmed with an external validation cohort.

Keywords: head and neck (H&N) cancer, salivary gland (SG) tumors, radiomics, MRI, DWI

INTRODUCTION

Salivary gland tumors represent about 3-6% of head and neck tumors, with different incidences among tumor histotypes (1). Imaging is commonly used to determine the anatomic origin of the lesions (superficial vs. deep) and the extent of the tumor, in the differentiation between benign and malignant lesions and in the evaluation of neck nodes. This information is crucial to defining the treatment plan, which highly depends on the histology of the tumor. For example, a superficial parotidectomy is performed in cases of pleomorphic adenomas when sited in the superficial portion of the gland, while a total parotidectomy is performed in cases of malignant tumors, and conservative management is the preferred choice for Warthin's tumors with low potential for malignancy (2).

Fine needle aspiration cytology with or without ultrasonography is an important technique for the pre-surgical evaluation the salivary gland masses. However, considering the rarity and variety of salivary gland neoplasms, particularly malignant lesions, this technique requires great experience and may be inconclusive due to inadequate samples (1, 2).

Ultrasonography (US) and magnetic resonance imaging (MRI) are useful in the evaluation of parotid gland tumors (3, 4). Morphologic features of the lesion can help to separate benign from malignant lesions, including the shape, margins, signal characteristics on T1-weighted and T2-weighted (T2-w) images, type of contrast enhancement (CE), and perineural spread (4, 5). The apparent diffusion coefficient (ADC) derived from diffusion-weighted imaging (DWI) and the enhancement pattern from dynamic contrast-enhanced MRI have also been demonstrated to improve the ability to discriminate benign and malignant lesions (6).

Although the use of multiparametric imaging has increased in recent years, the results are controversial in regard to the role of morphologic and functional parameters derived from multimodal MRI in the differential diagnosis of parotid gland tumors (4, 5, 7, 8). Some studies indicate that sharp margins do not indicate malignancy (7, 8), while others found that heterogeneous CE cannot be used to distinguish benign from malignant lesions (4). An overlap of the mean ADC values between low-grade malignant lesions and benign lesions has also been described (4, 9).

Radiomics is a rapidly emerging field that was proposed a few years ago to extract mineable quantitative features from medical images such as CT, MR, and PET-CT images *via* dedicated algorithms and methodologies (10). The outputs of these analysis are parametric variables that could be correlated with genomic and clinical parameters, particularly in oncologic applications, which provide a more comprehensive tumor description and improve diagnostic accuracy and clinical predictions (11).

Innumerable radiomic features can be calculated in relation to the shape, pixel intensity histogram, and distribution of pixel intensities inside or in the neighborhood of a region of interest (texture analysis), which are potentially useful in predicting the pathological characteristics, response to treatment, and overall survival (12, 13). The purpose of this study is to evaluate the role of MRI-based radiomic analysis using both T2-w images and ADC maps in the differentiation of parotid lesions, and to develop predictive models with validation using an external patient cohort.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board and was conducted in accordance with the ethical statements of the Declaration of Helsinki. The requirement for informed consent was waived by the institutional review board. This study involved a retrospective evaluation of MRI examinations of 69 patients with parotid gland lesions, consecutively identified in our Institute between 2015 and 2019.

Histopathology diagnosis was obtained in all cases on surgical specimens, by a pathologist who is dedicated to the evaluation of head and neck tumors and has more than 10 years of experience. The exclusion criteria were: recurrence, unsatisfactory image quality, lesions with diameter <5 mm to avoid bias due to partial volume effects.

All patients underwent pre-treatment MRI studies. The patient group included 41 men and 28 women with an average age of 61.1 ± 14.8 years (range 27-90 years). A total of 69 parotid lesions were evaluated, of which 37 were benign, including 13 (18.8%) Warthin's tumors and 18 (26.1%) cases of pleomorphic adenoma. The other 32 lesions were malignant. Of the 10 parotid metastases, six were from previous cutaneous squamous cell carcinoma and four from previous cutaneous melanoma. The patient and tumor characteristics are provided in more detail in **Table 1**.

TABLE 1 | Patients' characteristics of training and validation cohort.

Characteristic	Training cohort	External Validation cohort
Patient Number	69	44
Age (years)		
Mean ± standard deviation	61.1 ± 14.8	57.5 ± 15
Sex (male/female)	41/28 (59.4%/ 40.6%)	26/18 (59.1%/40.9%)
Tumor type, n (%)	69 (100%)	44(100%)
Benign	37 (53.6%)	24 (38.6%)
Pleomorphic adenoma	18 (26,1%)	17 (38.6%)
Basal cell adenoma	2 (2.9%)	-
Adenomyoepithelioma	1 (1.5%)	-
Myoepithelial	2 (2.9%)	-
Oncocytoma	1 (1.5%)	-
Warthin tumor	13 (18,8%)	7 (15.9%)
Malignant	32 (36.4%)	20 (45.5%)
Mucoepidermoid carcinoma	5 (7.2%)	3
Acinic cell carcinoma	2 (2.9%)	3
Ductal carcinoma	4 (5.8%)	3
Adenoidocystic carcinoma	6 (8.7%)	4
Lymphoepithelial carcinoma	3 (4.4%)	7 others (3 high grade, 1 cystadenocarcinoma, 3 myoepithelial)
Carcinoma ex pleomorphic adenoma	1 (1.5%)	
Squamous cell carcinoma	1 (1.5%)	
Metastasis	10 (14.5%)	

This population was used as a training cohort and divided into three groups: benign tumors with the exclusion of Warthin's tumors (24 cases), Warthin's tumors (13 cases), and malignant tumors (32 cases). Three predictive models were built to compare these groups in pairs. Furthermore, another 44 patients were recruited at the Department of Radiology of the University of Brescia (Italy) and used as an external validation cohort. The tumor characteristics of this cohort are reported in **Table 1**.

MRI Acquisition Protocols

In Rome, for the training cohort, MRI was performed on a 1.5-T system (Optima MR 450w, GE Health-care, Milwaukee, WI, USA) with dedicated 16-channel receive-only radiofrequency coils: a head coil, a surface neck coil, and a spine coil. The MRI examination included fast spin-eco (FSE) T2-weighted images on the coronal plane (acquisition matrix 288 × 256, field of view 27 x 27 cm, TR/TE 5901 ms/102, slice thickness 4 mm). Next, axial FSE T2-weighted images were obtained (TR/TE 6844 ms/105 ms, field of view 26 cm, in-plane spatial resolution 0.47 mm × 0.47 mm, slice thickness 3 mm, spacing between slices 3.3 mm) along with pre-contrast T1-weighted images (acquisition matrix 288 × 256, field of view 20 cm, TR/TE 617 ms/8.1, slice thickness 3 mm) on the axial plane, which were acquired from the level of the skull base to the thoracic inlet.

DWI was obtained *via* single-shot spin-echo and echo-planar imaging (field of view 26–28 cm, in-plane spatial resolution 2-2.2 mm × 2-2.2 mm TR/TE 4500 ms/77 ms, slice thickness 4 mm, spacing between slices 5 mm, bandwidth 1953 Hz/pixel). Three different b values were used (b = 0, 500, and 800 s/mm^2) with diffusion-sensitizing gradients applied in three orthogonal directions to obtain trace-weighted images. ADC maps of the training set were generated using the commercial software package Ready View (GE Advantage Workstation, READYView, Palo Alto, CA, USA). The imaging protocol also included post-contrast (Gadolinium 0.1 mmol/kg) T1-w images with liver acquisition with volume acceleration (LAVA) sequences (acquisition matrix 288 × 288, field of view 26-26 cm, TR/TE 9.8 ms/min, slice thickness 1 mm, 214 slices) in axial and coronal planes as required for the routine examination.

In Brescia, for the validation cohort, MRI was performed on a 1.5-T system (Aera, SIEMENS Healthineers Medical Solutions, Knoxville, TN, USA) with dedicated head and neck coils. The parameters of T2-w images were similar to those used for the training cohort (TR/TE 52 20 ms/105 ms, in-plane spatial resolution 0.43 mm × 0.43 mm, slice thickness 3 mm, spacing between slices 4.5 mm). DWI was obtained *via* single-shot spin-echo and echo-planar imaging (field of view, 25 cm in-plane spatial resolution 1.8 mm × 1.8 mm, TR/TE 3900 ms/60 ms, bandwidth 1455 Hz/pixel, slice thickness 3 mm, spacing between slices 4.5 mm). Two different b values were used (b = 50 and 800 s/mm^2) with diffusion-sensitizing gradients applied in three orthogonal directions to obtain trace-weighted images. ADC maps of the validation cohort were automatically generated by the software MR Syngo (SIEMENS, Healthineers Medical Solutions).

Extraction of Radiomic Features

The extraction of the radiomic features was performed using S-IBEX software (14). S-IBEX is a standardized version of IBEX (image biomarker explorer) software (15) that was recently adapted and validated according to the guidelines of the Image Biomarker Standardization Initiative (IBSI) (16). The entire tumor volume was delineated by consensus between two radiologists with more than 20 and 10 years of experience in head and neck (A.V. and F.P) using T2-w images.

First- and second-order features were derived from a volumetric analysis of T2-w images, including morphological features (29 features), intensity histogram features (23 features), intensity-volume histogram features (7 features), and grey level co-occurrence matrix or GLCM (25 features). Only first-order features from the intensity direct analysis (9 features) were extracted from ADC maps for a total of 93 features for each lesion. The IBSI reference manual (16) suggests not using some morphological features because they do not have reference values (i.e., the minimum volume enclosing ellipsoid volume and area density, as well as the oriented minimum bounding box volume and area density). Thus, these four features were not included in the statistical analyses, leaving a total of 89 features that were finally evaluated for each lesion.

A description of each feature family is reported in **Supplemental Data**. The formulas used for the calculation are described the IBSI reference manual (16). Details on the image pre-processing, including interpolation, re-segmentation and intensity discretization, are indicated in **Supplemental Data**.

Qualitative Evaluation of Margins and Contrast Enhancement Type

Two radiologists who have more than 10 years of experience in head and neck and were unaware of the pathological results examined all pre-surgery MRI examinations in relation to the type of margins (regular if the lesion border was well-defined in any sequence or irregular if the lesion border was ill-defined) on both T2- and T1-w images, and the type of CE (1 homogeneous, 2 inhomogeneous, 3 absent) in post contrast T1-w images. The results were obtained by establishing a consensus between the radiologists. The qualitative scores were also included in the feature selection and model building.

Statistical Analysis

The feature selection and modeling were performed in the Matlab environment. The relationships between categorical variables (type of CE and margins) and the classification response were evaluated using the chi-squared test. The initial selection of the most significant features was carried out using the Mann-Whitney test with a cutoff for p of 0.10. Before further selection of the remaining features, the training and validation datasets were standardized using the z-score normalization method as indicated by Haga et al. (17). Based on this method, each feature was normalized as z=(x-)/std, where x, and std are the feature value, mean value, and standard deviation, respectively. Thus, a *neighborhood component analysis (NCA)* was applied through the Matlab function *fscnca* to further reduce

the number of significant variables. To perform NCA, the regularization parameter lambda was tuned to find the optimal lambda value that produces the best classification performance.

In the case of high correlation between the selected features (Spearman correlation coefficient Rho >0.7, p <0.05), the one with the highest accuracy was chosen. The model with the final feature set was achieved using the support vector machine (SVM) binary classification algorithm. A five-fold cross-validation was applied to avoid overfitting due to the small dataset. The classification performance is reported in terms of the accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

RESULTS

The volumes of benign, Warthin's, and malignant lesions were 2.7 cm^3 (range, 0.2-21.1 cm^3), 5.2 cm^3 (range, 0.6-69.2 cm^3), and 5.1 cm^3 (range, 0.5-114 cm^3), respectively.

Relevant features included in the predictive models are reported in **Table 2**. The predictive performance of the three models on the training cohort and those tested on the validation cohort is reported in **Tables 3** and **4**, respectively. In the training cohort, the model for discriminating between Warthin's and malignant tumors reached the best accuracy of 86.7% (sensitivity 87.5%, specificity 84.6%) with a combination of four parameters: the 25th percentile of ADC (P25), the morphological feature of the volume density of the approximate enclosing ellipsoid (AEE) from T2-w images, and the type of margins and enhancement. When this model was tested on the validation cohort, it produced an accuracy of 77.8% (sensitivity 90%, specificity 42.9%).

In the training cohort, the model for discriminating between benign and Warthin's tumors showed a high accuracy of 91.9% (sensitivity 84.6%, specificity 95.8%) with a combination of four parameters: P25 of ADC, volume density AEE, minimum histogram gradient from T2-w images, and the type of enhancement. When this model tested on the validation cohort, it produced a comparable accuracy of 91.7% (sensitivity 85.7%, specificity 94.1%).

In the training cohort, the model for discriminating between benign tumors and malignant tumors had an accuracy of 80.4% (sensitivity 84.4%, specificity 75%) with a combination of four

TABLE 2 | Relevant features included in the predictive models.

	Warthin's Tumors		Malignant Tumors		*P value**
	Median	***IQR***	***Median***	***IQR***	
P25 of ADC (× 10^{-6} mm^2/s)	911	190	1058	379	0.054
Volume Density AEE	1.29	0.07	1.26	0.10	0.011
	Benign Tumors		Warthin's Tumors		
	Median	*IQR*	*Median*	*IQR*	*P value**
P25 of ADC (× 10^{-6} mm^2/s)	1506.88	612.00	911.00	189.75	<0.001
Volume Density AEE	1.26	0.07	1.29	0.07	0.0481
Minimum Histogram Gradient	-7.25	15.25	-16.00	18.63	0.0582
	Benign Tumors		Malignant Tumors		
	Median	*IQR*	*Median*	*IQR*	*P value**
P25 of ADC (× 10^{-6} mm^2/s)	1507	612	1058	379	<0.001
P10 of T2	9.00	3.00	6.50	4.00	0.007

**P values refer to Mann-Whitney test. P25, 25th percentile of the ADC distribution inside the lesion; P10 of T2, 10th percentile of the T2-weighetd signal intensity distribution inside the lesion; AEE, approximate enclosing ellipsoid.*

TABLE 3 | Predictive Performance of the three models on the training cohort.

End-point	Selected Features	Accuracy(%)	Sensitivity(%)	Specificity(%)	PPV(%)	NPV(%)
Warthin's versus Malignant Tumors	*ADC P25* *Volume Density AEE* Margins Gd	86.7 [73.2, 95.0]	87.5 [71.0, 96.5]	84.6 [54.5,98.1]	93.3 [79.5, 98.1]	73.3 [51.7, 87.6]
Benign* versus Warthin's Tumors	*ADC P25* *Volume Density AEE* *MinimumHistogramGradient* Gd	91.9 [78.1, 98.3]	84.6 [54.6, 98.1]	95.8 [78.9, 99.9]	91.7 [61.4, 98.7]	92.0 [76.2, 97.6]
Benign* versus Malignant Tumors	*ADC P25* *T2 P10* Gd Margins	80.4 [67.6, 89.8]	84.4 [67.2, 94.7]	75.0 [53.3, 90.2]	81.8 [68.9, 90.1]	78.2 [60.9, 89.3]

**Benign tumors with exclusion of Warthin's tumors. Abbreviations as in previous tables. In squared brackets the 95% confidence interval is reported.*

TABLE 4 | Predictive Performance of the three models tested on the validation cohort.

End-point	Selected Features	Accuracy(%)	Sensitivity(%)	Specificity(%)	PPV(%)	NPV(%)
Warthin's versus Malignant Tumors	*ADC P25*	81.5	90.0	57.1	85.7	66.7
	Volume Density AEE	[61.9,93.7]	[68.3,98.8]	[18.4,90.1]	[71.6,93.5]	[31.7, 89.6]
	Margins					
	Gd					
Benign* versus Warthin's Tumors	*ADC P25*	91.7	85.7	94.1	85.7	94.1
	Volume Density AEE	[73.0,99.0]	[42.1,99.6]	[71.3, 99.9]	[46.7,97.6]	[72.2, 99.9]
	MinimumHistogramGradient					
	Gd					
Benign* versus Malignant Tumors	*ADC P25*	89.2	85.0	94.1	94.4	84.2
	T2 P10	[74.6,97.0]	[62.1,96.8]	[71.3,99.9]	[71.6,99.1]	[65.1, 93.8]
	Gd					
	Margins					

**Benign tumors with exclusion of Warthin's tumors. Abbreviations as in previous tables. In squared brackets the 95% confidence interval is reported.*

parameters: P25 of ADC, P10 from T2-w images, and the types of margins and of CE. When this model was tested on the validation cohort, it produced an accuracy of 89.2% (sensitivity 85%, specificity 94.1%).

The results of chi-squared tests performed on the qualitative variables (type of margins and type of CE) included in the model building are reported in **Table 5**. **Figure 1** shows three correct classified lesions in the training dataset and three misdiagnosed cases in the validation set (a Warthin's tumor, a pleomorphic adenoma, and a malignant tumor, respectively).

The values of the most significant features initially selected by the Mann-Whitney test for each group and box plots of the features finally included in the models are shown in **Supplemental Data**.

TABLE 5 | Chi-square Test performed on qualitative variables, Type of Margins (a) and Type of Contrast Enhancement (b) in the three patient groups.

a. Type of Enhancement	Homogeneous	Inhomogeneous	Absent	P value
Warthin's Tumors	0	7	6 (28.9%)	
Malignant Tumors	1	25	6 (71.1%)	0.151
	(2.2%)	(71.1%)	(26.7%)	
Benign Tumors	11	12	1 (64.9%)	
Warthin's Tumors	0	7	6 (35.1%)	0.001
	(29.7%)	(51.4%)	(18.9%)	
Benign Tumors	11	12	1 (42.9%)	
Malignant Tumors	1	25	6 (57.1%)	0.0004
	(21.4%)	(66.1%)	(12.5%)	

b. Type of Margins	Irregular Margins	Regular Margins	P value
Warthins' Tumors	0	13 (28.9%)	0.009
Malignant Tumors	19	13 (71.1%)	
	(42.2%)	(57.8%)	
Benign Tumors	4	20 (64.9%)	0.315
Warthins' Tumors	0	13 (35.1%)	
	(10.8%)	(89.2%)	
Benign Tumors	4	20 (42.9%)	0.003
Malignant Tumors	19	13 (57.1%)	
	(41.1%)	(58.9%)	

DISCUSSION

In the evaluation of parotid gland tumors, there is overlap of the imaging signs between different neoplastic histologies (6, 8, 18), which represents a major limitation in the pre-surgical work-up of these lesions. Some recent studies report that texture analysis of MRI may provide a useful and objective description of signal patterns, which contribute to accurate diagnosis between tumors that look alike by a visual inspection (11, 13). The value of a computer-assisted discrimination of benign and malignant tumors has been explored in various organs (19–21), but only a few studies have assessed the contribution in parotid masses (12, 22–26).

In the present investigation, we identified the most discriminative features from pre-surgery MRI examinations based on first- and second-order texture analyses of T2-w images and first-order texture analysis of ADC maps for the separation of benign and malignant parotid lesions. All the three proposed models had good to excellent predictive performance, in combination with qualitative scores related to the type of margins or CE. This suggests that the texture analysis should be used as an additional tool for supporting radiologists' decisions and not in isolation (22).

Consistent with prior studies, we found significantly lower ADC values for Warthin's tumors than those of benign and malignant tumors (27, 28). Among the ADC-derived parameters, the 25th percentile (P25) of the ADC distribution inside the lesion was found to be the most relevant and was selected in all three models. This confirms the important role of DWI for the differential diagnosis of parotid lesions, as reported in previous studies (4, 9, 22, 29, 30). The P25 of ADC represents the ADC value associated with the tumor sub-volume with the most

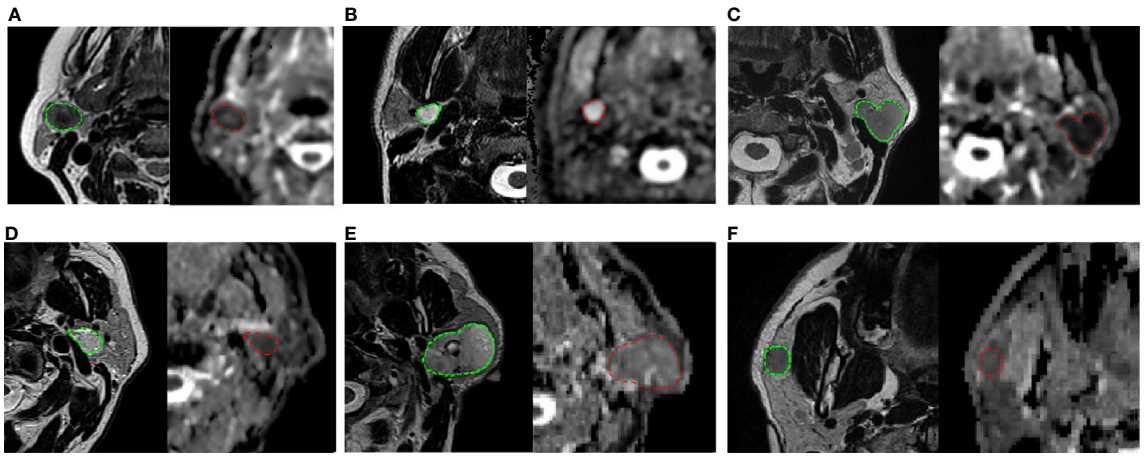

FIGURE 1 | On the top: three correctly classified lesions in the training dataset: **(A)** Warthin's tumor with low T2 intensity, ovoidal shape and decreased ADC value (P25 of ADC = 0.834 × 10^{-3} mm^2/s), **(B)** pleomorphic adenoma with typical T2 hyperintensity, sharp margins and high ADC value (P25 of ADC is 1.693 × 10^{-3} mm^2/s) **(C)** malignant tumor with irregular margins, T2 hypointensity and low ADC value, (P25 = 0.744 × 10^{-3} mm^2/s). At the bottom: three misdiagnosed cases in the validation set: **(D)** Warthin's tumor with high T2 hyperintensity and irregular shape (P25 of ADC = 0.930 × 10^{-3} mm^2/s); **(E)** pleomorphic adenoma with no typical T2 intensity and low ADC value (P25 of ADC = 1.109 × 10^{-3} mm^2/s); **(F)** malignant tumor with typical very low T2 intensity but regular and sharp margin and ovoidal shape (P25 of ADC = 0.836 × 10^{-3} mm^2/s). Each frame illustrates T2-weighted axial image with the user-defined lesion contour on the left and the corresponding ADC map on the right.

restrictive water molecule mobility. Thus, it is potentially related to a tumor region with a higher cell density. This finding suggests that instead of mean/median ADC values, it would be preferable to use a histogram-based approach to better address the tissue heterogeneity inside the tumor, which typically characterizes both benign and malignant parotid lesions (6, 8, 18). In this context, an interesting study of Khalek Abdel Razek et al. (31) evaluated the added value of Diffusion Tensor Imaging (DTI) to differentiate subtypes of parotid tumors, on the basis of fractional anisotropy (FA) and mean diffusivity, reporting very high accuracies. Even though DTI cannot be considered part of routine head and neck oncologic protocols, it showed a great potential to accurately separate Warthin's tumors from malignant tumors, as well as Warthin's tumours among all the other benign tumors. In particular, FA appeared to be associated to the complexity and heterogeneity of tissue microstructure and it may provide deeper insights into the parotid tumor cytoarchitecture, compared to conventional ADC.

The volume density AEE derived from T2-w images showed discriminatory potential for separating Warthin's tumors from other parotid tumors. This feature is directly related to the volume sphericity and showed increased values for Warthins' tumors, indicating that this kind of lesion has a more spherical shape than both malignant and benign tumors ($p = 0.025$ and $p = 0.04$ respectively, Mann-Whitney test). Concerning T2-w images, P10 was found to be relevant for differentiating benign and malignant lesions, with the latter showing significantly lower P10 values ($p = 0.007$, Mann-Whitney test) according to previous studies (5, 19, 23, 32, 33). In fact, it was demonstrated that high, intermediate, and low signal intensity can be associated with benign lesions (pleomorphic adenoma), intermediate, and highly malignant tumors, respectively (5, 32). Therefore, the use of heavily T2-w sequences is strongly suggested (33).

Recently, Sarioglu et al. (12) reported a texture-based study of T2-w images and contrast-enhanced T1-w images to discriminate the most common parotid tumors, in addition to several qualitative scores, as we similarly proposed. Due to the small number of malignant parotid tumors included and to differences in MR sequences considered for the analysis, a direct comparison with our findings is not possible. However, the authors showed that both skewness and kurtosis were significantly different between pleomorphic adenoma, Warthin's tumors, and mucoepidermoid carcinoma. In the present study, the skewness was also found to be discriminative for the separation of benign and malignant/Warthin's lesions, even though it was not included in the final model. The role of the minimum histogram gradient from T2-w images in the model is less obvious for differentiating benign and Warthin's tumors. This feature was strongly associated with several GLCM-based features, such as the dissimilarity, contrast, and inverse difference (Spearman's coefficient Rho = 0.894, 0.870, and -0.906 with $p < 0.0001$), with the latter being a measure of homogeneity (16). Therefore, an increased value of the minimum histogram gradient in the group of benign lesions should suggest higher contrast and inhomogeneity compared to the group of Warthin's tumors.

The explanation for this phenomenon is not straightforward, considering that Warthin's lesions typically show high tissue heterogeneity (22, 27). In fact, the tissue contrast of this kind of lesion can be affected by degenerative alterations in the interstitial tissue and may depend on the degree of differentiation of tumor cells, as well as the presence or

absence of necrosis and cystic components (27, 34). This causes a broad range of MR signal intensity, which reflects the variable proportion of microcytic components and lymphoid stroma inside the lesion (34). On the other hand, benign lesions may also show tissue heterogeneity on T2-w images due to cystic, solid, or mixoid components. Moreover, it was recently reported that the volume of the lesion may impact the value of some T2-w radiomic features, such as dissimilarity and energy, as shown by Wormald et al. (35). They found that larger cervical cancers had lower dissimilarity and higher energy and thus higher homogeneity and uniformity than smaller ones. In our dataset, Warthin's tumors showed a tendency to be larger (median, 5.2 cm^3) than benign lesions (median, 2.7 cm^3), even though there was no statistically significant difference between these volumes ($p = 0.22$, Mann-Whitney test). This may partially explain our findings.

The macroscopic imaging signs involved in the models (i.e., the type of margins and CE) were previously found to be useful in differential diagnosis (12). In fact, an ill-defined tumor border and low-grade contrast enhancement were observed as independent risk factors for malignancy, while a well-defined tumor margin was reported as a good qualitative indicator of benignity (12, 23).

The potential role of contrast enhancement and perfusion in discriminating various subtypes of parotid tumors was specifically addressed by some previous studies, which proposed the use of arterial-spin labeling (ASL) (30), dynamic susceptibility contrast perfusion-weighted MRI or dynamic contrast enhanced MRI (2, 4, 36, 37). These investigations consistently indicated that Warthins's tumors are characterized by a higher tissue vascularity than pleomorphic adenomas, and generally by a lower vascularity than malignant tumors. Although further efforts should be made to improve the repeatability and reproducibility of perfusion-weighted techniques (38) before including them as part of routine head and neck oncologic protocols, they are very promising and merit future investigation.

Lastly, we tested the performance of the developed prediction models on an external validation dataset, which is strongly suggested for a complete radiomic analysis to verify the reproducibility and transportability in a clinical setting (10). The predictive performance on the validation cohort indicated comparable accuracies, even though the model discriminating between Warthin's and malignant tumors showed lower specificity and negative predictive power (they decreased from 86.6 to 57.1, and from 73.3 to 66.7%, respectively).

This is consistent with a recent study of Gabelloni et al. (25), who also proposed a radiomic analysis of parotid tumors on T2-w MR images and obtained the best classification performance when comparing benign tumors with Warthin's tumors, while a lower accuracy was found in differentiating Warthin's and malignant tumors. Radiologists have particular difficulties in differentiating this type of lesion, the reason is that it may present a solid component with low signal intensity in T2-w images, which is also found in malignant lesions. Furthermore, they may have cystic components with low and high signal intensity in T1-w images, which indicate the presence of cystic fluid or high protein fluid, respectively (2, 27, 39). As mentioned, DWI has the potential to appreciably improve this misclassification, as Warthin's tumors typically show lower ADC values than both benign and low grade malignant tumors (22, 27).

Recent literature has shown a growing interest in the clinical applicability of radiomics for the parotid tumor characterization (12, 25, 26), thanks to significant improvements in diagnostic accuracy obtained with a multiparametric approach to quantitative MRI (2, 4, 6, 30, 31, 36, 37). However, no consensus exists regarding the most appropriate sequences to consider for the extraction of radiomics features and only a few studies used standardized software, previously validated according to the updated IBSI guidelines (16). Moreover, the lack of an external validation set in most of current papers makes impossible to verify the transportability of the proposed models. In general, further efforts are needed for a standardization of the entire workflow, from image acquisition and processing to feature extraction, statistical analysis and clinical validation (40). This could facilitate a direct comparison between findings from single centers, helping to clarify the added role of MRI-based radiomics in oncologic applications.

The present study has some limitations. First, its retrospective nature may have introduced bias and confounding factors. Secondly, our findings should be confirmed in a larger patient population as only a small number of benign and malignant tumors were included in the training cohort. Another limitation is the lack of differentiation between low and high-grade lesions in the context of malignant neoplasms due to the low number of patients, which could allow us to develop specific predictive models as a function of tumor grade.

CONCLUSIONS

Radiomic analysis of ADC and T2-w images in addition to qualitative scores evaluating margins and CE allowed us to obtain good to excellent diagnostic accuracies in differentiating parotid lesions, which was confirmed by testing on an external validation cohort.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, Regina Elena National Cancer Institute (IRCCS), Rome, Italy. Written informed consent for participation was not required for this

study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FP and AV conceived of the study and drafted the manuscript. SM carried out the radiomic analysis and contributed to the draft. RP contributed to the patient enrollment and data gathering. RCo provided the histological data on surgical specimens. VF and FP carried out the image delineation and participated in the image analysis. MR, DF, and RCa provided the external validation dataset and contributed to the design of the study. IT performed the statistical analysis. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.656918/full#supplementary-material

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"Fistula Zero" Project After Total Laryngectomy: The Candiolo Cancer Institute Experience

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Objectives: Pharyngocutaneous fistula (PCF) is a troublesome complication after total laryngectomy. The "Fistula zero" project aims to reduce the number of PCF by following a detailed protocol based on three fundamental key points.

Materials and Methods: The Fistula zero project included 77 patients who underwent total laryngectomy in the period from January 2019 to December 2020. The protocol consisted of three main aspects: the systematic placement of a Har-El salivary bypass tube, the continuous horizontal watertight pharyngeal suture using a barbed suture, onlay insetting of a pedicled flap in pre-treated patients.

Results: One case of PCF (1.3%) and three small blind fistulas (3.9%) were observed in this series. The mean length of hospitalization was 18 days.

Conclusion: Pharyngocutaneous fistula (PCF) prolongs hospitalization and delays adjuvant treatments. Thanks to a strict adherence to the protocol, it was possible to reduce PCF rates, avoiding lengthy hospitalization and additional surgical procedures.

Keywords: laryngeal cancer, total laryngectomy, pharyngocutaneous fistula, complications, Montgomery tube, bypass tube, pedicled flap, head and neck cancer

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INTRODUCTION

Total laryngectomy (TL) is considered the gold standard surgical treatment for advanced laryngeal cancer. Surgery can be performed as primary treatment or as salvage treatment after failure of previous surgical or non-surgical protocols.

Pharyngocutaneous fistula (PCF) is a common complication after TL, with an incidence ranging from 3% to 65% (1–4) [9–25% after primary surgery, 30–70% after salvage laryngectomy (5)]. When PCFs occur after total laryngectomy, several challenging consequences have to be expected: delays for adjuvant treatments, frequent need for revision surgery, an increase in the length of hospitalization, delays in the rehabilitation process leading up to oral food intake, reduction in quality of life, and higher costs (both social and economic).

Abbreviations: COPD, chronic obstructive pulmonary disease; PCF, pharyngocutaneous fistula; TL, total laryngectomy; NGT, naso-gastric tube; ND, neck dissection; TLM, transoral laser microsurgery; OPHL, open partial horizontal laryngectomy.

Many studies have focused on the risk factors associated with PCF (6, 7) and many surgical strategies have been proposed to reduce its incidence (3, 5, 8, 9). Of these, one is represented by the routine placement of a salivary bypass tube. Even though some interesting results related to this strategy are reported in the literature, robust evidence is still lacking (10, 11).

The main aim of this study is to present the results of the project, titled "Fistula zero after total laryngectomy."

MATERIALS AND METHODS

In total, 77 consecutive patients underwent primary/salvage TL at the Head and Neck Oncological Unit of the FPO IRCCS, Candiolo Cancer Institute, in the period from January 2019 to December 2020 and were included in this study.

All of the procedures performed were considered to be conventional in terms of technique and indications, according to the current guidelines, to the ethical standards of the Institutional and/or National Research Committee and to the 1964 Helsinki Declaration and its later amendments. Ethical review and approval were not required for this study in accordance with the national and institutional requirements. Before surgery, every patient signed a consent form for the disclosure of appropriate personal data for scientific purposes. Written informed consent was obtained from all of the patients. They all underwent the same clinical assessment during the 3 weeks before surgery, including clinical examination, nutritional status evaluation (body mass index [BMI]), biopsy/pathological examination, maxillofacial and neck MRI/CT scan, and total body PET scan. Two surgeons (GS and EC) carried out all of the procedures. Demographic data for the study population are summarized in **Table 1**.

The "fistula zero protocol" was adopted for the whole series. Following the protocol, a Har-El salivary bypass tube (Boston Medical™, Westborough, MA, USA) was placed in the neopharynx with a naso-gastric tube (NGT) positioned inside it before performing the pharyngeal closure; the bypass tube was secured by a stitch passing through the base of the tongue toward the skin, where it was knotted to prevent pressure ulcers (**Figures 1A–E**). The Har-El pharyngeal tube was chosen because of its particular funnel shape, designed to be easily anchored at the tongue base. The posterior aspect of the tube is higher while the anterior wall has a lower, flattened profile: this feature prevents it from being displaced upward into the oropharynx.

A watertight pharyngeal suture was performed using a continuous barbed suture (V-lock suture; Covidien™, Mansfield, VA, USA). Two resorbable stitches were placed at each end of the neopharynx to keep the mucosa stretched; two V-lock sutures were used to suture each hemipharynx as far as the midline. A second layer of reinforcement was then made with a third barbed suture. In pre-treated patients [radiotherapy (RT) with or without concomitant chemotherapy (CRT)], a pedicled flap (pectoralis major myofascial flap) was harvested and placed upon the pharyngeal suture.

Patients who had undergone earlier laryngeal surgical procedures [transoral laser microsurgery (TLM)/open partial horizontal laryngectomy (OPHL)] were not included in this group. This study focused on patients pre-treated with chemoradiotherapy, since such treatments may represent one of the main risk factors for postoperative fistula formation.

In every patient, a barium dynamic swallowing test with liquid and semi-liquid bolus was performed in both antero-posterior and lateral projections before commencing oral intake. The exam was performed on the 9th postoperative day in the group of patients where no reconstructive surgery was carried out, and on the 12th postoperative day in the group of patients who underwent reconstructive surgery by pectoralis major myofascial flap. In the case of absence of PCF, oral food intake started on the 10th postoperative day for patients who underwent only total laryngectomy without reconstruction, and on the 13th postoperative day for those who underwent reconstructive surgery. Oral feeding began with the salivary bypass tube in place and after 2 days, the patient was discharged. The NGT was removed on the same day as commencement of oral feeding and the salivary bypass tube was removed 1 week after the barium swallowing test (if it showed no fistulas).

When the barium swallowing test indicated the presence of a PCF/blind fistula, a compressive cervical dressing was put in place and oral feeding was delayed.

RESULTS

The fistula zero protocol was applied in 77 patients (70 men, 7 women) who underwent primary/salvage TL for laryngeal cancer; the median age was 67 years (range, 47–90 years).

In total, 27 patients (35.1%) were pre-treated [RT: 7 patients (9.1%), CRT: 20 patients (26.0%)]. Comorbidities were scored by applying the Adult Comorbidity Evaluation-27 Index (12). This score is composed of 12 organ system related categories and 27 subcategories; it aims to quantify a specific disease. The Overall Comorbidity Score was defined according to the highest ranked single disorder (0 = none, 1 = mild, 2 = moderate, and 3 = severe). More than two grade 2 scores gave an overall score of 3.

Nineteen patients (25%) were staged pT3: they were not amenable to OPHL or organ-sparing protocols due to important comorbidities (ACE-27 grade 2). Moreover, some of them had undergone previous chemoradiotherapeutic treatments for oropharyngeal squamous cell carcinoma/non-Hodgkin lymphoma. Four patients (5%) were very old (>80 years) and had severe comorbidities (ACE-27 grade 3): in these cases, we preferred to carry out a total laryngectomy.

The median intraoperative time for the surgical procedure was 210 min.

The salivary bypass tube demonstrated good patient-tolerability with no complications (bypass tube dislocation, migration, granulation tissue formation). The average length of hospitalization was 18 days (range, 12–40 days): 21 days for patients who underwent reconstruction and 16 days for those who did not.

TABLE 1 | Demographic data for the 77 patients in this study.

Characteristic	No. of patients (%)
Age, years	
Mean	67 ± 10
Range	47–90
Sex	
Male	70 (90.9)
Female	7 (9.1)
Comorbidities	
No	15 (19.5)
1 comorbidity	18 (23.4)
2 comorbidities	30 (38.9)
≥3 comorbidities	14 (18.2)
ACE-27 Grade 1	39 (50)
ACE-27 Grade 2	19 (24)
ACE-27 Grade 3	4 (5)
BMI	
Mean	24
Range	17–31
Pre-treatment	
Yes	27 (35.1)
No	50 (64.9)
Pathological status	
pT4a	27 (35.1)
pT3	19 (24.7)
pT2	4 (5.2)
ypT4	16 (20.8)
ypT3	6 (7.8)
ypT2	5 (6.5)
Neck dissection	
Ipsilateral	30 (39)
Pre-treated	12 (17)
Primary	18 (23)
Bilateral	40 (51.9)
Pre-treated	8 (10)
Primary	32 (41)
None	
Pre-treated	7 (9.1)
Thyroidectomy	
Hemi-thyroidectomy	20 (26)
Total thyroidectomy	27 (35.1)
Hystmectomy	30 (38.9)
Complications	
PCF	1 (1.3) [not-pre treated]
Minimum extraluminal spill of barium	3 (3.9) [2 pre-treated (2.5%), 1 primary patient (1%)]

PCF occurred in only one patient who had not been pre-treated (1.3%), who experienced more than three comorbidities (cardiopathy, diabetes, kidney failure, chronic obstructive pulmonary disease, ACE-27 grade 3) and who was treated by revision surgery (a pectoralis major myofascial flap was harvested and placed to cover the PCF). Three patients (3.9%), two pre-treated and one not pre-treated, developed a minimum extraluminal spill of barium (blind fistula), successfully managed with a compression dressing. In these cases, oral feeding was postponed. Donor-site complication (seroma) occurred in four pre-treated patients (5%) and was managed with a compression dressing.

DISCUSSION

Complications after TL are always associated with delayed adjuvant treatment, a longer hospitalization due to the delayed rehabilitation, and the need for additional postoperative surgical procedures. However, a trend toward a reduction in postoperative complications has recently been reported. In a systematic review of 522 studies (2177 patients), Sayles and Grant reported an incidence of 14.3% for pharyngocutaneous fistula in patients treated by primary laryngectomy with an increased incidence of 27.6% after salvage laryngectomy (3).

There are several risk factors related to PCF, which can be summarized as patient-related, disease-related and treatment-related. Concerning patient-related risk factors, age > 60 years, presence of > 1 comorbidity (lung disease, cardiopathy, diabetes), smoking status, nutritional status, low albumin level, and low hemoglobin level (7, 13–16) have to be considered. Disease-related risk factors are represented by advanced T stage and tumor site (supraglottic region) (17). Regarding treatment-related risk factors, previous treatments, such as RT/CRT, causing tissue fibrosis and skin necrosis, can lead to delayed mucosa healing, resulting in PCF. Neck dissection, requiring a longer operating time, could lead to an increased risk of wound infection and PCF formation. In addition, the skill of the surgeon, especially with regard to watertight closure of the pharyngeal wall, could represent a risk factor for PCF formation.

The "fistula zero protocol" was introduced to minimize the rate of PCF formation after TL, based on information from literature data. Many studies have analyzed the usefulness of the salivary bypass tube in preventing PCF formation, but the heterogeneity of patients included in those studies did not allow a meaningful meta-analysis to support strong evidence in clinical practice (11). The salivary bypass tube, placed for the first time in 1978 by Montgomery (18) to bridge the gap between the pharyngostome and esophagostome after laryngoesophagectomy, is recommended in high-risk patients to prevent PCF formation after total laryngectomy and to prevent stenosis of the cervical esophagus and tracheoesophageal fistula (11, 19).

More recently, Gilardi et al. precisely described how to manage the positioning of a salivary bypass tube with the help of a Cuffed-Reinforced Oral/Nasal Tracheal Tube, to prevent or treat some complications of laryngeal and hypopharyngeal surgery (20). Moreover, many authors have previously endorsed the application of a salivary pharyngeal tube, often in association with reconstructive surgery, to help wound healing in patients who developed PCF after laryngeal surgery (21–24).

Concerning the present study, the calyx-shaped Har-El pharyngeal tube allows better adaptability to the pharynx, allowing an efficient collection of saliva inside the tube, therefore reducing its spread over the pharyngeal suture.

On the other hand, based on literature data, closure of the pharyngeal wall could represent a risk factor for PCF formation. Several studies have demonstrated that a mechanical pharyngeal suture could represent an advantage in patients undergoing total laryngectomy, mainly for the reduction in PCF formation (8.7% with an absolute risk reduction of 15%). Nevertheless, the benefits offered by stapler-assisted closure could not be

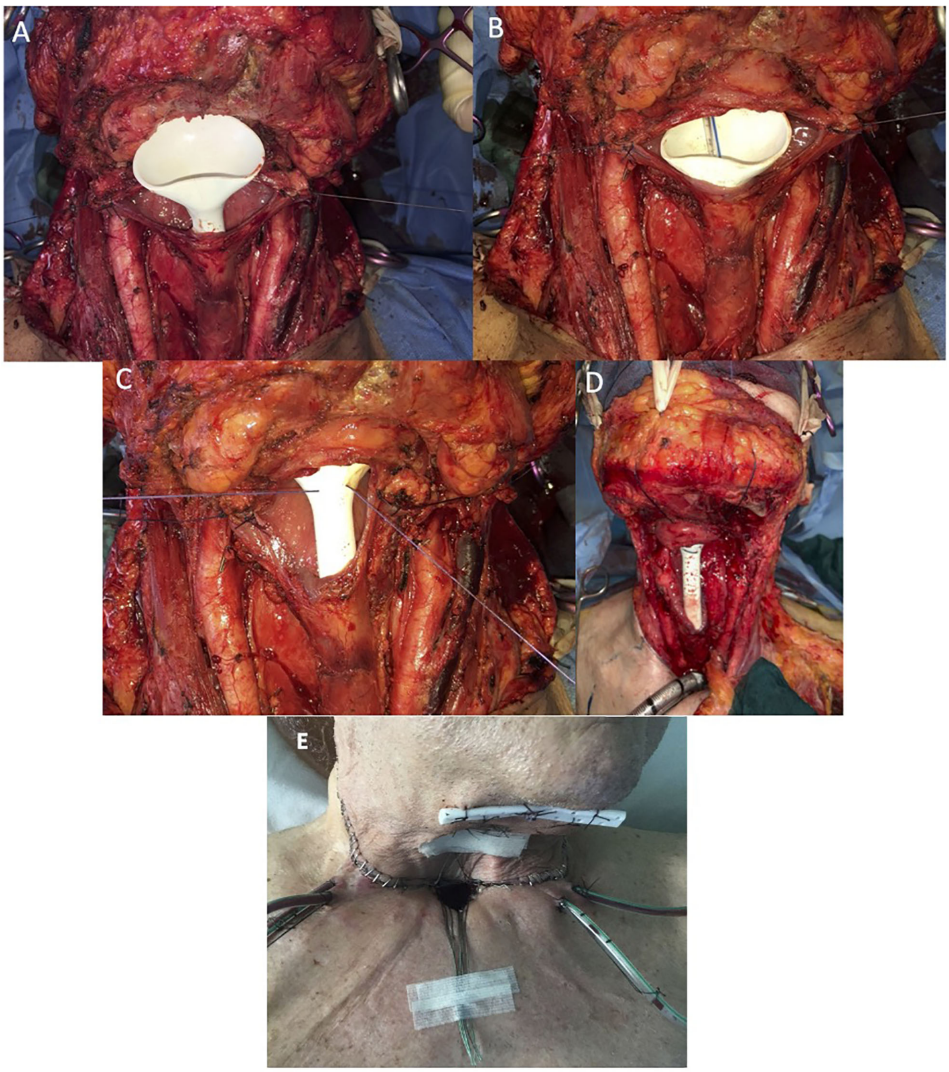

FIGURE 1 | **(A)** Intraoperative positioning of salivary bypass tube. **(B)** Naso-gastric tube (NGT) insertion inside salivary tube. **(C, D)** Transcutaneous securing of salivary tube. **(E)** Salivary stent fixation on the skin.

definitively identified among patients who had previously undergone organ-sparing protocols (25).

In the fistula zero protocol, the pharyngeal suture is performed horizontally using a barbed suture. Currently, three types of barbed suture are commercially available: the Quill Self-Retaining System (SRS) (B. Braun Medical Ltd, Sheffield, UK), the bidirectional barbed suture (Angiotech, Vancouver, BC, Canada) and the V-Loc unidirectional barbed suture (V-lock suture; Covidien™, Mansfield, VA, USA) (26–28). The barbed stitches allow for a continuous suture, without tension, reducing the possibility of knots slipping, secondary dehiscence to knot breakage, extrusion or suture splitting and necrosis caused by knot compression on tissues. Furthermore, a continuous suture without knots gives a better seal against liquids, reducing the potential infiltration of saliva between tissues of different thickness and consistency. Three stitches are normally used to complete the tension-free suture.

The third key point of our project is using a pedicled flap to reinforce the pharyngeal suture in pre-treated patients (radiotherapy with or without concomitant chemotherapy). The onlay of a pedicled/free flap in pre-treated patients is now widely supported in the literature, because of the radiation-induced microvascular injury that leads to hypovascular, hypocellular, and hypoxic tissue. Many authors suggest that reinforcing the pharyngeal suture with well-vascularized tissue will help to reduce the incidence of PCF formation in these patients (5, 29, 30). The pectoralis major myofascial flap is one of the most reliable flaps, but many other reconstructive options are described (supraclavicular artery island flap, fasciocutaneous free flaps, mammary artery

perforator propeller flap, latissimus dorsi flaps, and facial artery-based cutaneous island flap) (21, 22, 31–34).

In our practice, a pectoralis major myofascial flap was sutured over the pharyngeal suture, without compressing it. The systematic and rigorous adoption of our protocol allowed us to observe excellent results in terms of minimal complications (1.3%), especially when comparing pre-treated (1.3%) and untreated (3.9%) patients, showing similar low rates of complications, also stratifying the patients on the basis of patient- and disease-related risk factors.

The management of PCF has a huge economic impact. Parikh et al. estimated that 57% of patients who develop a fistula require surgical revision. They reported postoperative complications in 22% of patients, with a final cost, which included hospitalization and surgical procedures, of about $58 000 for each fistula (35).

The results of this study seem to be encouraging from both a clinical and economic perspective. In our cohort, the mean hospitalization time was 18 days (range 12–40 days). Hospitalization was longer (21 days) in pre-treated patients (reconstruction; donor-site seroma occurred in four patients and was managed with compression dressing) and shorter (16 days) for patients who did not undergo reconstruction. Higher surgery-related costs (mean € 550 for each procedure) are balanced by a reduction in the length of hospitalization and the absence of delayed adjuvant therapy and oral feeding.

The strengths of this study are represented by the relatively large series and uniformity of surgical procedures. The main limitation of the study is the lack of a control group of patients not treated with this protocol. The preliminary results obtained in this study encourage us to propose a multi-institutional perspective study to validate the usefulness of the Har-El salivary bypass tube in preventing PCF after TL.

CONCLUSION

Sustainability of medical care, especially in oncology, is a delicate and debated topic, especially in a world where comorbidities increase with increasing age of the population. More standardized procedures are required, and total laryngectomy represents an excellent model from this point of view.

"Fistula zero" is undoubtedly an ambitious project (there is no surgery without complications) but not an unapproachable strategy. Thanks to a careful attitude and a meticulous approach, the project achieved very low rates of pharyngocutaneous fistula and small blind fistula formation.

The results of this study were obtained by rigid adherence to the protocol and uniformity of surgical procedure and suggest that, with a small increase in surgical costs, it is possible to reduce overall costs for PCF management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EC, conception and design of the study, surgeon, writing and editing the manuscript. GA, data collection. AS, medical statistician who performed statistical analysis. GS, conception and design of the study, surgeon, editing the manuscript. All authors contributed to the article and approved the submitted version.

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Predictive Value of Skeletal Muscle Mass in Recurrent/Metastatic Head and Neck Squamous Cell Carcinoma Patients Treated With Immune Checkpoint Inhibitors

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Background: Reduced muscle mass has been associated with increased treatment complications in several tumor types. We evaluated the impact of skeletal muscle index (SMI) on prognosis and immune-related adverse events (IrAEs) in a cohort of recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) treated with immune checkpoints inhibitors (ICI).

Methods: A single-institutional, retrospective study was performed including 61 consecutive patients of R/M HNSCC diagnosed between July 2015 and December 2018. SMI was quantified using a CT scan at L3 to evaluate body composition. Median baseline SMI was used to dichotomize patients in low and high SMI. Kaplan-Meier estimations were used to detect overall survival (OS) and progression-free survival (PFS). Toxicity was recorded using Common Terminology Criteria for Adverse Event v4.3.

Results: Patients were 52 men (85.2%) with mean of age 57.7 years (SD 9.62), mainly oral cavity (n = 21; 34.4%). Low SMI was an independent factor for OS in the univariate (HR, 2.06; 95% CI, 1.14–3.73, p = 0.017) and multivariate Cox analyses (HR, 2.99; 95% CI, 1.29–6.94; p = 0.011). PFS was also reduced in patients with low SMI (PFS HR, 1.84; 95% CI, 1.08–3.12; p = 0.025). IrAEs occurred in 29 (47.5%) patients. There was no association between low SMI and IrAEs at any grade (OR, 0.56; 95% CI, 0.20–1.54; p = 0.261). However, grades 3 to 4 IrAEs were developed in seven patients of whom three had low SMI.

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Conclusions: Low SMI before ICI treatment in R/M HNSCC patients had a negative impact on OS and PFS. Further prospective research is needed to confirm the role of body composition as a predictive biomarker in ICI treatment.

Keywords: head and neck (H&N) cancer, body composition, muscle mass, immune checkpoint inhibitors, sarcopenia, immune-related adverse events (irAE)

INTRODUCTION

Among patients with head and neck squamous cell carcinoma (HNSCC), between 5% and 10% are diagnosed with metastatic disease. Additionally, despite aggressive multimodal strategies, about 60% of patients treated with radical intention for a locally advanced disease will eventually recur (1). Until the introduction of immunotherapy agents, the median survival was 10.1 months, with an 82% rate of grades 3 to 4 adverse events using the historic standard first-line EXTREME (combining platinum and 5-fluorouracil (5-FU) and cetuximab) (2). Patients with progressive disease after platinum-based chemotherapy have a poor prognosis with a 1-year survival under 5% (3). Hereby, there is an urgent need for improved therapy in the recurrent and metastatic (R/M) population.

Targeting the programmed cell death (ligand)-1 (PD-(L)1) pathway has shown significant activity, and improved overall survival (OS) in patients with previously treated R/M HNSCC, associated with fewer grades 3 or 4 toxicities than standard therapy (4, 5). These results have led to approval of two anti-PD1 agents (pembrolizumab and nivolumab) as second-line treatment for patients with R/M HNSCC who experience disease progression on or after a platinum-based therapy (6, 7). More recently, pembrolizumab has been approved in the first-line setting, alone or in combination with chemotherapy (8). Despite improving the results compared with older strategies, approximately 70% of patients do not benefit from immune checkpoint inhibitors (ICI) as they have progression as the best response, enhancing the need for predictive biomarkers (6, 8).

Patients with R/M HNSCC are at an increased nutritional risk, and malnutrition has been shown to be an independent indicator of prognosis in cancer patients (9). The nutritional deterioration of HNSCC patients is often present from diagnosis and worsens throughout onco-specific treatments (10). This deterioration does not only occur exclusively at the expense of weight alone but also because the loss of muscle mass has been shown to associate with prognosis and complications (10, 11).

Sarcopenia, defined as a reduced skeletal muscle mass that reduce muscle function, is noted in geriatric populations (12). Reduced muscle mass is also prominent in patients at any age with different chronic diseases, including cancer. This is also termed sarcopenia, and it is typically classified in relation to the risk of disease-specific outcomes, such as mortality, surgical complications, or cancer treatment (13). Sarcopenia has been reported to have a significant impact on both OS and complications in cancer patients undergoing onco-specific treatment and/or surgery (14). These results have also been described in head and neck cancer patients (15–18). Although some studies have revealed that low muscle mass may also have a role in the oncological outcomes in patients with melanoma (19, 20) or lung cancer (21, 22) treated with ICI, as far as we know, there are no current studies evaluating the impact of low muscle mass in R/M HNSCC undergoing these therapies.

We aim to evaluate the muscle mass as a predictive biomarker of OS and progression-free survival (PFS) in patients diagnosed with R/M HNSCC treated with ICI. A secondary analysis focused on the association of muscle mass on the onset of immune-related adverse events (IrAES).

MATERIALS AND METHODS

Population and Study Design

This longitudinal retrospective single-center study was approved by the local ethics committee for clinical research (PR302/18). All patients provided written informed consent. Patients diagnosed with R/M HNSCC treated with ICI, regardless of treatment line, from July 2015 and December 2018 at the Catalan Institute of Oncology, were evaluated. Patients were eligible if they had R/M HNSCC and were treated with ICI including anti-PD1 or anti-PDL1 alone or in combination with other ICI (such as anti-CTLA4) or chemotherapy and had a staging full-body computed tomography (CT) scan as part of their pre-treatment procedure (within 10 days prior to the introduction of ICI) and at evaluation of tumor response according to RECIST criteria, version 1.1 (23).

Clinical data included age, sex, smoking status, alcohol consumption, Eastern Cooperative Oncology Group Performance Status (ECOG-PS), TNM on Cancer (7th edition) (24), primary tumor site, treatment line for R/M disease, type of recurrence prior ICI, and response. Those patients, who had received the last dose of platinum 6 months before of initiation of ICI, were classified as platinum-refractory. Additional information regarding IrAEs according to the CTCAE version 4.3 2009 (25) and vital status were also collected from medical records.

Nutritional data were collected at baseline (before starting treatment). These data included body mass index (BMI calculated as [(weight (kg)/height (m^2)], serum albumin levels, and type of nutritional intervention if any.

Image Analysis

All treatment images were selected by a trained researcher to ensure they correspond to the same vertebra landmarks to allow a proper comparison of body composition. Values were obtained by a single observer blinded to the patients' data.

Images were accessed from the axial cross-sectional CT as all patients had an abdominal CT scan as part of their routine care. The third lumbar (L3) vertebra was chosen on the axial cross-section CT component of the full-body CT scans as the reference point, based on previous reports with this level to calculate the skeletal muscle index (SMI) (26, 27) using SliceOmatic© software (v5.0 Rev 8, Tomovision, Montreal, Quebec, Canada). Regional analysis at L3 strongly predicted whole-body fat and fat-free mass (r=0.86-0.94; p < 0.001) (26). Muscle cross-sectional area (CSA) was quantified within a Hounsfield unit (HU) range from -29 to +150HU and then normalized for height to report as SMI (cm^2/m^2). Sarcopenia was defined according to Martin L et al. (28) using specific SMI cut points for advanced cancer patients. CSA of adipose tissue were determined using tissue-specific HU range defined at this level (29).

Statistical Analysis

To define cohort characteristics, categorical variables were presented as the number of cases and percentages, whereas continuous variables were presented as the mean and standard deviation (SD) or median and interquartile range (IQR). Median baseline SMI was used to dichotomize patients in two groups: low SMI (patients with baseline SMI lower than median baseline SMI) or high SMI (patients with baseline SMI equal or higher than median baseline SMI).

It was planned to test for the effect of baseline SMI on survival. Time between treatment initiation and disease progression or death from any cause (PFS) and time between treatment initiation and death from any cause (OS) was assessed using the Kaplan-Meier estimator. One-year OS rate and 1-year PFS rate were also analyzed. The Cox proportional hazards model was used to perform univariate and multivariate survival analyses, which are reported as the hazard ratio (HR) and 95% confidence interval. Covariates with a p-value lower than 0.1 in the univariate model were included in the multivariate models. The proportionality of risks in the Cox model was verified using the Schoenfeld residuals.

To evaluate the effect of baseline variables in the development of toxicity, logistic regression models were used. Odds ratios and their corresponding 95% confidence intervals were derived from both univariate and multivariate models.

Statistical significance was set at a probability level ≤0.05. The statistical package used to treat the data and perform the statistical analysis was R software version 3.5.

RESULTS

Patient Characteristics

Table 1 shows baseline demographic and clinical characteristics of the 61 patients included in the analysis. Most patients were male (n = 52, 85.2%) with a mean age of 57.7 years (SD 9.62). Tumor location was mainly oral cavity (n = 21; 34.4%). Most of patients recurred with locoregional plus metastatic disease (n = 28; 45.9%). Four (6.5%) patients received ICI as the first treatment and 59% (n = 36) were platinum refractory.

At baseline, the mean BMI was 23.8 kg/m^2 (SD 4.56); underweight (BMI ≤18.5) was present in 9 (14.8%) patients and 26 (42.6%) were overweight (BMI ≥25) or obese (BMI ≥30). Median SMI was 42.0 cm^2/m^2 (IQR 37.5; 48.6) and was used to classify patients between high and low SMI. Nutritional support was required in 34 (55.7%) patients, 15 (44.1%) of them needed a tube feeding. Two thirds (n=41, 67.2%) of the patients were sarcopenic according to previously published cut points (30) and three of them were also obese (**Table 1S**, **Supporting Information**).

Significant differences were identified for patients with low *vs* high SMI in mean age (p = 0.035), baseline weight (p < 0.001), BMI (P < 0.001), and total adipose tissue (p = 0.003). Patients with high SMI were older, heavier, and with higher BMI. No other significant differences between patients with low and high SMI at baseline were found.

Effects of SMI in Overall Survival (OS) and Progression Free Survival (PFS)

Median follow-up time was 9 months (range, 3.6–21.3). Up to a third of patients (n=16; 26.2%) were alive at last follow-up. The median time to death was 4.3 months (range, 2.3–10.9). **Table 2** summarized univariate and multivariate analyses of OS, PFS, 1-year survival, and 1-year PFS.

Patients with low SMI had shorter OS (HR, 2.06; 95% CI, 1.14–3.73; p = 0.017) (**Figure 1**) and 1-year OS rate (HR, 2.64; 95% CI, 1.33–5.23; p = 0.005) in the univariate analysis. Low SMI was also associated with global PFS (global PFS HR, 1.84; 95% CI, 1.08–3.12; p = 0.025, and 1-year PFS rate HR, 1.83; 95% CI, 1.01–3.23; p = 0.036) among other factors such as age (PFS HR, 0.96; 95% CI, 0.94–0.99; p = 0.002), baseline albumin (PFS HR, 0.95; 95% CI, 0.91–0.99; p = 0.027), platinum-refractory (PFS HR, 3.04; 95% CI, 1.67–5.56; p = <0.001), and any number of prior lines for R/M disease (PFS HR, 1.94; 95% CI, 1.09–3.46; p = 0.025). The association was maintained at 1-year PFS, although the number of prior lines showed only a trend (PFS HR, 1.71; 95% CI, 0.93–3.16; p = 0.084). One-year PFS showed a clear association with age (PFS HR, 0.97; 95% CI, 0.94–1.00; p = 0.036), serum albumin (PFS HR, 0.94; 95% CI, 0.90–0.99; p = 0.025), low SMI (PFS HR, 2.53; 95% CI, 1.19–5.37; p = 0.015), and patients who received platinum within 6 months prior to ICI (PFS HR, 3.57; 95% CI, 1.50–8.51; p = 0.004).

The multivariate analyses adjusted for serum albumin, baseline SMI, and platinum-refractory confirmed low SMI as an independent predictor for OS (HR, 2.19; 95% CI, 1.19–4.05; p = 0.012) and 1-year survival (HR, 2.79; 95% CI, 1.37–5.67; p = 0.005) adjusting this analysis also for age. Type of recurrence prior ICI initiation and BMI were not included in the multivariate analysis because it did not show association for survival or PFS in the univariate analysis.

Similar results were found using previously published cut points for sarcopenia (28) for OS but not for PFS. These results showed that sarcopenia was an independent factor for OS (HR, 2.06; 95% CI, 1.01–4.23; p = 0.048) after adjusting by the same covariates than the analysis performed for low SMI. This analysis

TABLE 1 | Patient baseline characteristics (overall and according to low *vs* high skeletal muscle index) (SMI) (n=61).

	Overall (n=61)	Low SMI (n=30)	High SMI (n=31)	p-overall
Age, years				
Mean (SD)	57.7 (9.62)	55.1 (9.93)	60.3 (8.73)	0.035
Median (range)	59.0 (23-78)	55.9 (23-70)	61.3 (35-78)	0.753
Male, n (%)	52 (85.2)	24 (80.0)	28 (90.3)	0.301
Smoking status, n (%)				0.394
Current	28 (45.9)	16 (53.3)	12 (38.7)	
Former*	26 (42.6)	12 (40.0)	14 (45.2)	
Never	7 (11.5)	2 (6.7)	5 (16.1)	
Alcohol consumption#, n (%)				
Yes	36 (59.0)	19 (63.3)	17 (54.8)	0.699
Location, n (%)				0.283
Oral cavity	21 (34.4)	11 (36.7)	10 (32.3)	
Hypopharynx	8 (13.1)	5 (16.7)	3 (9.7)	
Larynx	19 (31.3)	6 (20.0)	13 (41.9)	
Oropharynx**	13 (21.3)	8 (26.7)	5 (16.1)	
Type of recurrence				0.700
Locoregional	23 (37.7)	10 (33.3)	13 (41.9)	
Distance	10 (16.4)	6 (20.0)	4 (12.9)	
Locoregional + distance	28 (45.9)	14 (46.7)	14 (45.2)	
Line of therapy, n (%)				0.865
First	22 (36.1)	10 (33.3)	12 (38.7)	
Second or above	39 (63.9)	20 (66.7)	19 (61.3)	
Type of ICI therapy, n (%)				0.786
AntiPD1	8 (13.1)	5 (16.7)	3 (9.7)	
AntiPD1+virus	1 (1.64)	0 (0.0)	1 (3.3)	
AntiPD1 + chemotherapy	3 (4.92)	2 (6.7)	1 (3.2)	
AntiPDL1	12 (19.7)	5 (16.7)	7 (22.6)	
AntiPDL1+antiCTLA4	22 (36.1)	11 (36.7)	11 (35.5)	
AntiPDL1+IOA	15 (24.6)	9 (29.0)	6 (20.0)	
ECOG-PS, n (%)				0.363
0	1 (1.64)	0 (0.0)	1 (3.2)	
1	58 (95.1)	30 (100)	28 (90.3)	
2	2 (3.28)	0 (0.0)	2 (6.5)	
Platinum within 6 months of ICI,n (%)	36 (59.0)	18 (60.0)	18 (58.1)	1.000
Weight, kg				
Mean (SD)	67.3 (15.0)	59.5 (10.9)	74.9 (14.7)	<0.001
Median [Q1; Q3]	65.2 [54.3;79.0]	58.8 [51.0;65.6]	77.5 [64.3;84.5]	<0.001
BMI, kg/m^2				
Mean (SD)	23.8 (4.56)	21.3 (3.33)	26.2 (4.32)	<0.001
Median (range)	23.6 (15.8-34.7)	21.7 (15.8-27.6)	26.8 (17.7-34.6)	<0.001
BMI categorized, kg/m^2				0.001
Underweight (<18.5)	9 (14.8)	7 (23.3)	2 (6.5)	
Normal (18.5 – 25)	26 (42.6)	17 (56.7)	9 (29.0)	
Overweight /obese (>25)	26 (42.6)	6 (20.0)	20 (64.5)	
Albumin, g/L				
Mean (SD)	42.9 (60.3)	43.0 (3.24)	42.9 (7.86)	0.933
Median [Q1; Q3]	44.0 [41.0;46.0]	43.0 [41.0;45.0]	44.0 [42.0;46.5]	0.302
SMI, cm^2/m^2				
Mean (SD)	43.6 (7.75)	37.2 (3.14)	49.8 (5.44)	<0.001
Median [Q1; Q3]	42.0 [37.5;48.6]	37.5 [35.2;39.6]	48.6 [46.1;53.0]	<0.001
TATI, cm^2/m^2				
Mean (SD)	91.4 (53.3)	71.7 (44.5)	111 (54.7)	0.003
Median [Q1; Q3]	98.8 [49.4;118]	70.8 [31.5;101]	112 [82.0;148]	0.004

**Ex-smoker defined as no cigarettes for more than 6 months before diagnosis.*

#Alcohol consumption defined as sustained heavy drinker (≥4 drinks per week in women and ≥5 drinks per week in men). Includes active and former drinkers.

ICI, immune checkpoint inhibitor; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; BMI, body mass index; SMI, skeletal muscle index; TATI, total adipose tissue index; IOA, immuno-oncology agent.

***3 of them HPV-related.*

is provided as **Supplementary Information** (**Table 2S** and **Figure 1S**).

We sought to determine whether different BMIs were associated with any of the abovementioned outcomes. There were no statistically significant differences in OS and PFS, when examining overweight or obese patients (BMI ≥ 25 kg/m^2) compared with patients with normal BMI.

TABLE 2 | Univariate and multivariate analysis examining OS, one-year survival, global PFS and one-year PFS in association with skeletal muscle index (SMI) (n=61).

Univariate analysis	**SURVIVAL**						**PROGRESION FREE SURVIVAL**					
	Overall survival			**1-year survival**			**Global PFS**			**1-year PFS**		
	HR	***95% IC***	***P value***	***HR***	***95% IC***	***P value***	***HR***	***95% IC***	***P value***	***HR***	***95% IC***	***P value***
BMI	0.97	0.91;1.04	0.432	0.96	0.89;1.03	0.232	0.96	0.91;1.02	0.237	0.96	0.90;1.03	0.235
Age	0.98	0.96;1.01	0.177	0.98	0.95;1.00	0.077	0.96	0.94;0.99	0.002	0.96	0.94;0.99	0.002
Serum albumin	0.97	0.93;1.00	0.056	0.97	0.93;1.01	0.112	0.95	0.91;0.99	0.027	0.95	0.90;0.99	0.024
Low skeletal muscle index	2.06	1.14;3.73	0.017	2.64	1.33;5.23	0.005	1.84	1.08;3.12	0.025	1.83	1.01;3.23	0.036
Platinum-refractory	1.76	0.94;3.28	0.075	1.85	0.91;3.78	0.089	3.04	1.67;5.56	<0.001	2.95	1.57;5.55	0.001
Type of recurrence												
Distance	0.86	0.35;2.11	0.748	0.77	0.28;2.15	0.625	0.80	0.36;1.77	0.582	0.71	0.28;1.78	0.466
Locorregional + distance	1.16	0.61;2.22	0.651	1.08	0.53;2.19	0.830	1.03	0.58;1.83	0.929	1.11	0.61;2.02	0.736
Line of therapy 2 or above	1.26	0.68;2.34	0.470	1.21	0.60;2.41	0.596	1.94	1.09;3.46	0.025	1.71	0.93;3.16	0.084
Multivariate analysis*	***HR***	***95% IC***	***P value***	***HR***	***95% IC***	***P value***	***HR***	***95% IC***	***P value***	***HR***	***95% IC***	***P value***
Age				0.99	0.96;1.02	0.359	#	#	#	0.97	0.94;1.00	0.026
Serum albumin	0.96	0.93;1.00	0.052	0.96	0.93;1.00	0.082	#	#	#	0.94	0.90;0.99	0.016
Low skeletal muscle index	2.19	1.19;4.05	0.012	2.79	1.37;5.67	0.005	#	#	#	1.90	1.04;3.48	0.037
Platinum-refractory	1.74	0.92;3.30	0.090	1.73	0.84;3.56	0.138	#	#	#	3.31	1.40;7.83	0.006
Line of therapy 2 or above							#	#	#	0.68	0.30;1.53	0.349

OS, overall survival; PFS, progression free survival; BMI, body mass index; ICI, immune checkpoint inhibitors; Type of recurrence includes locorregional disease, distance disease and locorregional+distance disease; Line of therapy includes first vs second or above lines.

**Adjusted for the covariates with p-value <0.1 in the univariate analysis.*

#Multivariate models for global PFS were not computed due to the small numbers of patients in the no-event group.

Treatment Toxicity

IrAEs occurred in 29 (47.5%) patients mainly in those treated with anti-PDL1 plus IOA (n = 15; 80%) and treated with anti-PD1 plus chemotherapy (n = 2; 66.7%). Thyroiditis, skin, and liver alterations were the most common IrAEs, and the vast majority was grade 1 or grade 2. Only seven patients developed grade 3 or above toxicity, three of them with low SMI.

There was no association with low SMI and IrAEs of any grade (OR, 0.56; 95% CI, 0.20–1.54; p = 0.261).

Different factors were examined to determine their effect in the development of IrAEs. Patients with BMI ≤18.5 kg/m^2 (OR, 0.09; 95% CI, 0.00–0.63; p = 0.012), the presence of distance metastasis (OR, 4.93; 95% CI, 1.01–30.4, p = 0.048), and those patients platinum-refractory (OR, 0.33; 95% CI, 0.11–0.94, p = 0.037) were associated with toxicity of any grade. In the multivariate analysis, only being refractory to platinum (OR, 2.88; 95% CI, 1.05–8.98, p = 0.050) was a predictive factor of IrAEs occurrence. The presence of distant metastasis was not included in the multivariate analysis because only three patients with distant metastasis did not develop IrAEs.

DISCUSSION

Immunotherapy is significantly changing the therapeutic landscape for R/M HNSCC (4, 5). Its clinical efficacy varied among HNSCC patients, and there is a lack of accurate and effective predictive biomarkers. Low SMI is frequently encountered in HNSCC patients (10, 16). However, whether low SMI can be used as a predictive biomarker for ICI remains unknown, and the clinical data regarding the association between SMI and ICI efficacy are quite limited. To the best of our knowledge, this is the first study to assess the association between SMI and clinical outcomes of R/M HNSCC patients undergoing ICI therapy.

In our study, low SMI was confirmed to be an independent factor for reduced OS and 1-year survival after adjusting the model for relevant factors associated with clinical outcomes in HNSCC. These findings are in agreement with other studies performed in melanoma and lung cancer (19, 31). We did not assess mortality specific for cancer as only two patients died from another cause different from the primary cancer. Important variables such as age, serum albumin, refractoriness to platinum or the number of lines of therapy prior to ICI therapy are well-known predictive factors. However, body composition is often overlooked in clinical practice. BMI is not a good indicator of body composition as elevated BMI may hide a distribution of low muscle mass increasing the risk for adverse outcomes (19, 32). Moreover, muscle has been shown to be one of the strongest parameters associated with mortality in cancer patients (33) even when weight and BMI are included in the analysis.

There are numerous cut points values published for sarcopenia although none of them is yet definitive. Some of these cut points used OS as the outcome according to the SMI (27, 30). We have chosen Martin et al. (30) as their population is a large sample with advanced stage and includes all BMIs. Moreover, these cut points have been used in many previous publications, so readers can compare our results. As our cohort is a slightly distinct population, we also chose the median L3 SMI cut point to evaluate SMI as a predictive biomarker in R/M HNSCC.

Although the mechanism by which reduced SMI has a negative effect on the clinical efficacy of ICI remains unclear. New evidence shows that skeletal muscle cells, as an endocrine organ, may secrete specific cytokines that regulate immunity.

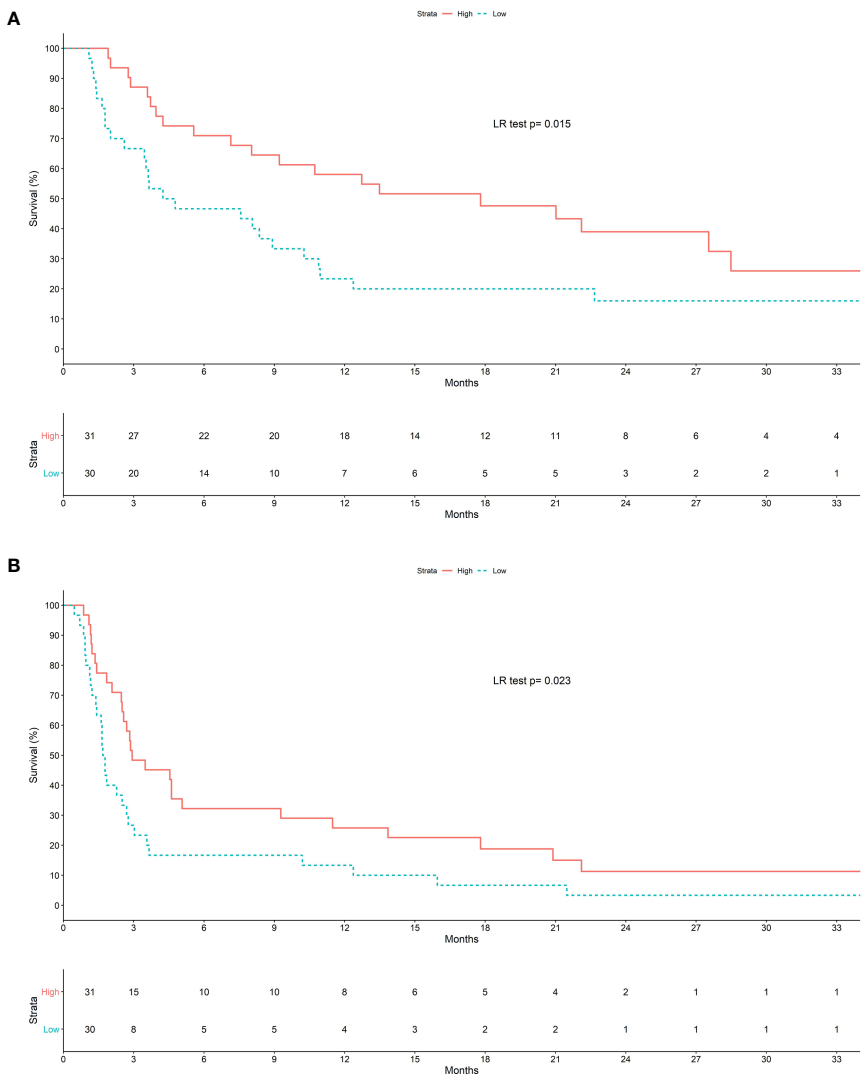

FIGURE 1 | Kaplan-Meier survival curves according to baseline SMI. **(A)** Overall survival. **(B)** Progression Free Survival.

These myokines are involved in modulating the immune response (34). Thus, a reduction in muscle mass may have a deleterious effect on the anti-tumor response mediated by the immune system, following in immunosuppression (35). A decrease in myokines due to the loss of muscle mass could suppress tumor response to ICI, resulting in the immune escape of tumor cells (36, 37). Inflammation also plays an important role in the loss of muscle mass (38). All these factors may contribute to the impairment of the antitumor immune response to ICI in HNSCC.

We did not find any statistically significant associations between BMI and clinical outcomes to ICI. Young et al. (19) identified trends toward worse outcomes in patients with high BMI and low muscle mass in patients with melanoma treated with anti-PD1. However, we included only six patients with BMI $\geq$ 25 kg/m^2 and low SMI.

Compared with traditional treatments (chemotherapy or radiotherapy), the incidence of toxicity in HNSCC patients treated with ICI has been reduced. We explored the effect of low SMI on the incidence of adverse event related to ICI in HNSCC patients, finding that low SMI was not significantly associated with the incidence of IrAEs. Evidence suggests that the incidence of IrAEs of any grade is associated with improved clinical outcomes (39). Unfortunately, subgroups analysis could not be further performed because of insufficient data.

Our study has some limitations that should be addressed. The main ones are the retrospective design of the study and the limited number of patients. Moreover, the CT imaging analysis was limited by the data availability; indeed, the acquisition protocol was planned according to the presence of previous examination. Finally, we did not take into account the type of ICI therapy alone or in combination.

In conclusion, our finding shows that baseline SMI is an independent factor for survival R/M HNSCC treated with ICI. SMI is not associated with the onset of IrAEs. Further prospective research is needed to confirm the role of body composition as a predictive biomarker in ICI treatment and how SMI can affect drug-specific and organ-specific adverse events caused by ICI in HNSCC patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: IDIBELL repository (http://diposit.ub.edu/dspace/handle/2445/172899).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Universitari de Bellvitge Ethics Committee for Clinical Research (PR302/18). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LA, MP, MT, RM, and VB were actively involved in the design of the stud writing the manuscript. LA, MP, MS, NV, AT, and LR were involved in the collection of data. NP and LA analyzed the data and all authors interpreted the data reviewed the manuscript. RM and VB supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.699668/full#supplementary-material

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Dual Inhibition of PARP and the Intra-S/G2 Cell Cycle Checkpoints Results in Highly Effective Radiosensitization of HPV-Positive HNSCC Cells

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In head and neck squamous cell carcinoma (HNSCC), tumors positive for human papillomavirus (HPV) represent a distinct biological entity with favorable prognosis. An enhanced radiation sensitivity of these tumors is evident in the clinic and on the cellular level when comparing HPV-positive and HPV-negative HNSCC cell lines. We could show that the underlying mechanism is a defect in DNA double-strand break repair associated with a profound and sustained G2 arrest. This defect can be exploited by molecular targeting approaches additionally compromising the DNA damage response to further enhance their radiation sensitivity, which may offer new opportunities in the setting of future de-intensified regimes. Against this background, we tested combined targeting of PARP and the DNA damage-induced intra-S/G2 cell cycle checkpoints to achieve effective radiosensitization. Enhancing CDK1/2 activity through the Wee1 inhibitor adavosertib or a combination of Wee1 and Chk1 inhibition resulted in an abrogation of the radiation-induced G2 cell cycle arrest and induction of replication stress as assessed by γH2AX and chromatin-bound RPA levels in S phase cells. Addition of the PARP inhibitor olaparib had little influence on these endpoints, irrespective of checkpoint inhibition. Combined PARP/Wee1 targeting did not result in an enhancement in the absolute number of residual, radiation induced 53BP1 foci as markers of DNA double-strand breaks but it induced a shift in foci numbers from S/G2 to G1 phase cells. Most importantly, while sole checkpoint or PARP inhibition induced moderate radiosensitization, their combination was clearly more effective, while exerting little effect in p53/G1 arrest proficient normal human fibroblasts, thus indicating tumor specificity. We conclude that the combined inhibition of PARP and the intra-S/G2 checkpoint is a highly effective approach for the radiosensitization of HPV-positive HNSCC cells and may represent a viable alternative for the current standard of concomitant cisplatin-based chemotherapy. *In vivo* studies to further evaluate the translational potential are highly warranted.

Keywords: head and neck cancer, human papillomavirus (HPV), molecular targeting, radiotherapy, radiosensitization, PARP, Wee1, Chk1

INTRODUCTION

In locally advanced squamous cell carcinoma of the head and neck (HNSCC), positivity for human papillomavirus (HPV) confers a favorable prognosis, especially for patients with tumors located in the oropharynx (OPSCC) (1, 2). Standard treatment of locally advanced disease is cisplatin-based chemoradiation, either in the primary setting or as adjuvant treatment after surgery. The combination of high cure rates but often dramatic toxicity under these regimes has resulted in the development of various clinical trials testing de-intensification approaches, and some early phase trials have reported promising results (3–7). Two phase 3 trials, however, which together recruited more than 1,000 patients, concordantly reported inferiority of the rather cautious de-intensification concept of exchanging cisplatin for the also approved anti-EGFR antibody cetuximab under maintenance of the full radiation dose (8, 9). In line with these negative clinical results, we had previously shown that cetuximab completely fails to radiosensitize HPV-positive HNSCC cells *in vitro* (10). This clearly urges caution and speaks in favor of careful preclinical evaluation of novel agents and concepts.

A way to very directly induce radiosensitization is the molecular targeting of proteins involved in the DNA damage response (DDR) and DNA repair. Poly(ADP-ribose) polymerase 1 (PARP1) is responsible for poly(ADP-ribose) polymerization at the sites of DNA damage, which marks the lesion and recruits further DNA repair factors. PARP1 is involved in single-strand break repair but also in double-strand break (DSB) repair *via* the alternative end-joining (alt-EJ) backup DSB repair pathway (11, 12). Sole PARP inhibition is especially effective in tumors with a severe deficiency in homologous recombination (HR). Following the well-known concept of synthetic lethality, PARP inhibition increases the need for effective HR by interfering with the repair of intrinsic single-strand lesions and PARP-trapping at the break sites. Upon collision with replication forks, these structures can lead to the formation of one-ended DSBs, the repair of which requires HR (13, 14). Ionizing radiation induces both single- and double-strand breaks, and PARP-inhibitors are well known radiosensitizers (15).

Cell cycle checkpoints constitute another important factor in the response towards irradiation, providing more time for DNA repair before entering S-phase or mitosis in order to avoid mutations and especially mitotic cell death (16). In HNSCC, the majority of HPV-positive and -negative tumors are functionally deficient for p53 and subsequently also for the G1-S cell cycle checkpoint, increasing the dependence on the G2-M checkpoint. Reduction of the radiation-induced G2 arrest can be achieved by inhibition of the ATR/Chk1/Wee1 axis, as the inhibition of any of these kinases finally counteracts Wee1-mediated inhibitory phosphorylation of cyclin dependent kinase 1 (CDK1), which, in its active state will continue to drive G2-M transition (16, 17). Premature mitotic entry and induction of severe replication stress are further therapeutic effects resulting from enhanced CDK1 and CDK2 activity upon inhibition of the ATR/Chk1/Wee1 axis also without irradiation (18–20).

We and others have demonstrated that PARP inhibition as well as inhibition of radiation induced cell cycle checkpoints *via* targeting of Chk1, ATR, or Wee1 can radiosensitize HPV-positive HNSCC cells (10, 21–25). Different mechanisms may account for the observed sensitization. HPV-positive HNSCC cells are described to rely on PARP-dependent alt-EJ (26, 27) and to be defective in homologous recombination (HR) (27–31). Due to an ineffective DSB repair, these cells further rely on an especially profound and long lasting radiation-induced G2 arrest for the repair of radiation-induced DSBs before the critical passage through mitosis (21, 22, 32, 33). Apart from interfering with G2 arrest, the inhibition of Wee1, Chk1, or ATR can directly compromise the ability to perform HR (34–36) and the induction of replication stress, which is to a large extent caused by nucleotide shortage due to unrestrained CDK activity and enhanced origin firing (18), that may create an unfavorable environment for DNA repair in S phase. Given these potential S/G2 phase-based mechanisms, it is easily imaginable that the combined inhibition of PARP and the S/G2 cell cycle checkpoints could be an especially effective treatment option for HPV-positive HNSCC cells, and its radiosensitizing effect has already been demonstrated in preclinical studies in a number of other cancer entities (37, 38). Against this background, we tested the combined inhibition of PARP and the S/G2 cell cycle checkpoint in intrinsically DSB repair-compromised HPV-positive HNSCC cells using clinically relevant inhibitors, all of which are already being tested in combination with radiotherapy in clinical trials in HNSCC.

MATERIAL AND METHODS

Cells and Cell Culture

All cell lines were grown in RPMI (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) at 37°C, 5% CO_2 and 100% humidification. HPV-positive HNSCC cells UD-SCC-2, UM-SCC-47 and UPCI-SCC-154, UPCI-SCC-90, 93VU-147T, UT-SCC-45, and normal human fibroblasts F184 were described previously (21, 33, 39). Tumor cell lines were identified by a short tandem repeat multiplex assay (Applied Biosystems, Waltham, MD, USA). PARP inhibition was performed using 1 μM olaparib (MyBiosource, San Diego, CA, USA). Wee1 inhibition was performed using 240 nM adavosertib (Selleckchem, Houston, TX, USA) and combined Wee1/Chk1 inhibition was performed at a dose of 60 nM adavosertib and 1 nM prexasertib (MedChemExpress, Monmouth Junction, NJ, USA) unless stated otherwise. Supplementation with nucleosides (EmbryoMax 100×, Sigma-Aldrich, St. Louis, MO, USA) was performed at a final dilution of 1/12.5.

Cell Proliferation

For cell proliferation analysis, cells were seeded into T25 cell culture flasks and after 4 h treated with inhibitors. The numbers of resulting cells were assessed after 5 days using a Coulter counter (Beckmann-Coulter, Brea, CA, USA).

Cell Cycle Assessment

Cells were harvested, fixed with 70% ethanol, briefly washed with PBS/0.2% Triton X-100, and subsequently incubated with PBS/

1% BSA/0.2% Triton X-100/DAPI (4′,6-Diamidin-2-phenylindol, 1 µg/ml) for 30 min at room temperature in the dark. Cells were washed once with PBS/0.2% Triton X-100, and flow cytometric analysis was performed using a MACSQuant10 with MACSQuantify Software (Miltenyi Biotec, Bergisch Gladbach, Germany). The proportion of cells in the respective cell cycle phases was calculated using ModFit LT™ software (Verity Software House, Topsham, ME, USA).

X-Irradiation

Cells were irradiated at room temperature with 200 kV X-rays (Gulmay RS225, Gulmay Medical Ltd., Suwanee, GA, USA; 200 kV, 15 mA, 0.8 mm Be + 0.5 mm Cu filtering; dose rate of 1.2 Gy/min).

DSB Reporter Gene Assay

Exponentially growing HNSCC cells containing stably integrated copies of the previously described GFP-based HR or NHEJ reporter plasmids pGC or pEJ (40) were transfected with an I-SceI expression vector for targeted DSB induction using Fugene HD (Promega, Fitchburg, WI, USA). Six hours post transfection, the medium was exchanged and supplemented with inhibitors or solvent (DMSO) as indicated, followed by another exchange plus supplementation 24 h post transfection. At 48 h post transfection, the cells were harvested and assessed for GFP expression by flow cytometry using a FACS Canto with FACS Diva software (Becton Dickinson, Franklin Lakes, NJ, USA). The gating of GFP-positive cells was set according to the negative control (Fugene HD + empty vector). Rates of DSB repair (% GFP-positive cells) were normalized to the respective transfection efficiency of the individual experiment as determined by parallel transfection with a GFP-expression vector (pEGFP-N1).

Immunofluorescence

Cells grown on glass cover slips were fixed with PBS/4% formaldehyde for 10 min, and permeabilized/blocked for 1 h or overnight with PBS/1% BSA/0.2%Triton X-100. The cells were subsequently incubated for 1 h at room temperature with the primary antibodies [mouse anti-53BP1 (clone BP13, Millipore, Billerica, MA, USA); rabbit anti-geminin (#10802-1-AP, Proteintech, Manchester, UK)] in blocking solution, washed four times with PBS/0.5% BSA/0.1% Triton X-100 before incubation with the secondary antibodies plus DAPI (1 µg/ml) and were then washed again four times before mounting with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells were inspected using an AxioObserver Z1 fluorescence microscope with ApoTome and Axiovision Software (Zeiss, Oberkochen, Germany). 53BP1 foci per nucleus were manually counted using stack images in maximum intensity projection. Nuclei with ≥20 foci were scored as "20".

Flow Cytometric Protein Quantification

Flow cytrometric measurement of relative protein staining intensity per cell in relation to the cell cycle phase was performed on either a FACS Canto with FACS Diva Software (Becton Dickinson, Franklin Lakes, NJ, USA) using FxCycle FarRed (Molecular Probes, Eugene, OR, USA) as nuclear counterstain or on a MACSQuant10 with MACSQuantify and Flowlogic Software (Miltenyi Biotec, Bergisch Gladbach, Germany & Inivai, Mentone Victoria, Australia) using DAPI as nuclear counter stain. In brief, cells were harvested, fixed with PBS/4% formaldehyde for 10 min, and then permeabilized and blocked with PBS/1% BSA/0.2% Triton X-100 for a minimum of 1 h. The cells were subsequently incubated (1 h; room temperature) with the primary antibody [rabbit anti-P-Histone3 (#06-570, Millipore, Billerica, MA, USA), mouse-anti-γ H2AX antibody (clone JBW301, Millipore, Billerica, MA, USA), and mouse anti-RPA32 (clone ME34, Santa Cruz, Santa Cruz, CA, USA)] in blocking solution, washed three times with PBS/0.5% BSA/0.1% Triton X-100 before incubation (1 h; room temperature) with the second antibody and were then washed again three times. DNA counterstaining was either performed with DAPI added to the secondary antibody or with FxCycle FarRed (Molecular Probes, Eugene, OR, USA) plus 300 ng/ml RNAse A and 0.2% Triton X-100 for 30 min at room temperature in the dark following the last washing step.

In case of RPA staining, the cells were pre-extracted after trypsinizaton by gentle resuspension (wide bore tips) of the harvested cell pellet in 500 µl ice cold PBS/0.1% Triton X-100/1 mM DTT followed by gentle shaking in horizontally placed reaction tubes on ice for 10 min. Afterwards, 1 ml cold PBS/1% BSA/1mM DTT was added, tubes were inverted several times, and the pre-extracted cells were collected in a pre-cooled centrifuge (5 min, 400 g). After discarding the supernatant, the pre-extracted cells were resuspended (wide bore tips) in PBS/4% formaldehyde and fixed for 10 min at room temperature before regular subsequent staining procedures as described above.

Colony Formation Assay

Radiosensitization was determined using delayed plating colony formation assay. Exponentially growing cells were treated with inhibitor and irradiated after 2 h of incubation. Twenty-four hours post irradiation the cells were seeded in defined numbers into T25 cell culture flasks without addition of inhibitors. Incubation time until colony formation varied between cell lines from 2 to 4 weeks; irradiated samples of HPV-positive cell lines were allowed to grow for an extended period of time, as colony formation was apparently delayed. The number of colonies containing more than 50 cells was assessed. In the case of UM-SCC-47, the cell number was adjusted to 5000 by addition of feeder cells (UM-SCC-47; 20 Gy) to support plating efficiency, and for UPCI-SCC-154 and F184 the medium was changed to a 1:1 mixture of RPMI/10% FBS and Amniomax C-100 medium/7.5% Amniomax Supplement (both Gibco, Thermo Fisher Scientific, Waltham, MA, USA)/7.5% FBS one (F184) or three (UPCI-SCC-154) weeks after seeding to facilitate colony formation.

Data Evaluation

Data analyses were performed using Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 6 (GraphPadSoftware, San Diego, CA, USA). All experiments were performed at least

three times, and single experiments always contained the full set of substances and radiation doses as indicated. Values presented are mean ± SD unless indicated otherwise. Two-tailed Student's t-test was used to assess statistically significant differences using GraphPad Prism 6.

RESULTS

To assess whether the dual inhibition of PARP and Wee1 may exert some additive or synergistic effects in HPV-positive HNSCC cells, we tested a combination of the PARP inhibitor olaparib and the Wee1 inhibitor adavosertib (MK-1775/AZD-1775) with regard to cell proliferation and cell cycle distribution. To this end we used individual inhibitor doses that previously demonstrated moderate effects on their own with regard to the respective cell lines and endpoints or a maximum concentration of 1 μM olaparib in the cell cycle analyses, which was previously proven sufficient to completely suppress the poly(ADP)-ribosylation of HPV-positive HNSCC cells upon H_2O_2 treatment (10, 22). Regarding proliferation we observed several statistically significant differences and the generally strongest reduction under combined inhibition but without a clear hint for a meaningful synergistic effect (**Figure 1A**). Regarding cell cycle distribution, adavosertib induced an accumulation of cells in the S-phase, indicative of replication stress, while olaparib had little effect on its own or when added to Wee1 inhibition (**Figure 1B**).

Radiation-Induced Cell Cycle Arrest

While the previous results did not indicate prominent synergistic effects, we further tested dual PARP and S/G2 checkpoint inhibition combined with ionizing irradiation. To assess a direct effect on the radiation-induced G2 arrest, we quantified the amount of phospho-histone H3 positive mitotic cells 5 h after 6 Gy ± inhibitor treatment (**Figure 2A**). Sole adavosertib treatment (240 nM) increased the rate of mitotic cells in two cell lines, indicating unscheduled mitotic entry upon Wee1 inhibition as previously described (41). Irradiation largely blocked mitotic entry in all strains irrespective of olaparib treatment (1 μM). Adavosertib completely suppressed this G2 arrest, except for UD-SCC-2 cells, where it could only partially override checkpoint execution (**Figure 2B**). Additionally testing a later time point of 8 h post irradiation, adavosertib treatment ± olaparib further relieved UD-SCC-2 cells from the radiation-induced G2 checkpoint (**Figure 2C**). We had previously shown that Wee1 inhibition activates Chk1, which could in part compensate the reduction in Wee1 activity and, indeed, dual inhibition was effective at profoundly reduced doses (22). As low dose dual Wee1/Chk1 inhibition may potentially offer a clinical alternative to high dose single inhibitor treatment, we also included a combination using especially low concentrations of 60 nM adavosertib and 1 nM of the Chk1/2 inhibitor prexasertib, which showed limited effectiveness on their own (**Supplementary Figure S1**). This dual checkpoint inhibition resulted in checkpoint abrogation comparable to the higher dose (240 nm) of sole adavosertib treatment irrespective of the addition of olaparib in all strains (**Figure 2B**).

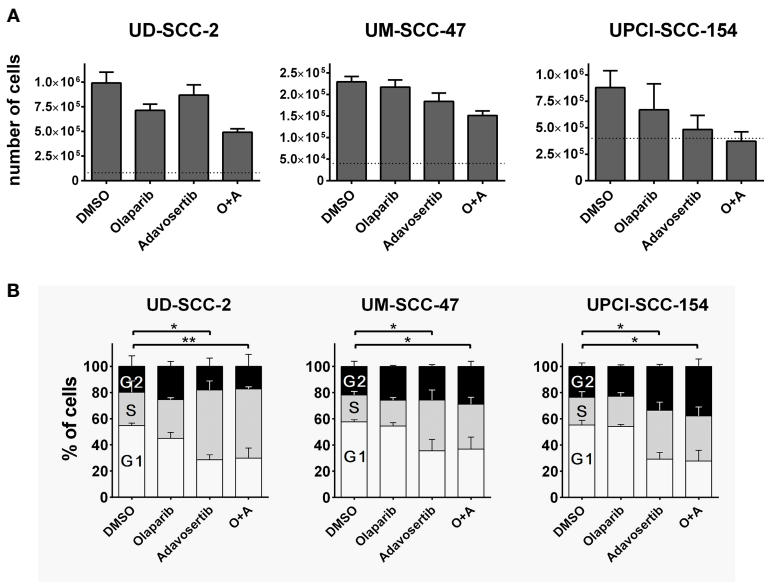

FIGURE 1 | Interactions of PARP and Wee1 inhibition. **(A)** Proliferation. Cells were seeded and after 4 h treatment with inhibitors as indicated. Five days later the respective numbers of cells were assessed. Dotted lines indicate the number of cells seeded. Adavosertib: UD-SCC-2 & UM-SCC-47, 120 nM; UPCI-SCC-154, 60 nM. Olaparib: all strains 500 nM. **(B)** Cell cycle. Cells were seeded and on the next day treated with the respective inhibitors. After 24 h, the cells were fixed and subjected to DAPI staining and flow cytometric assessment of cell cycle distribution. Adavosertib: UD-SCC-2 & UPCI-SCC-154, 480 nM; UM-SCC-47, 960 nM. Olaparib: all strains 1 μM. Statistical evaluation was performed for changes in the S-phase population; addition of olaparib did not induce any significant changes. Asterisks depict significant differences with * and ** indicating $p < 0.05$ and $p < 0.01$, respectively (two-tailed Student's t-test).

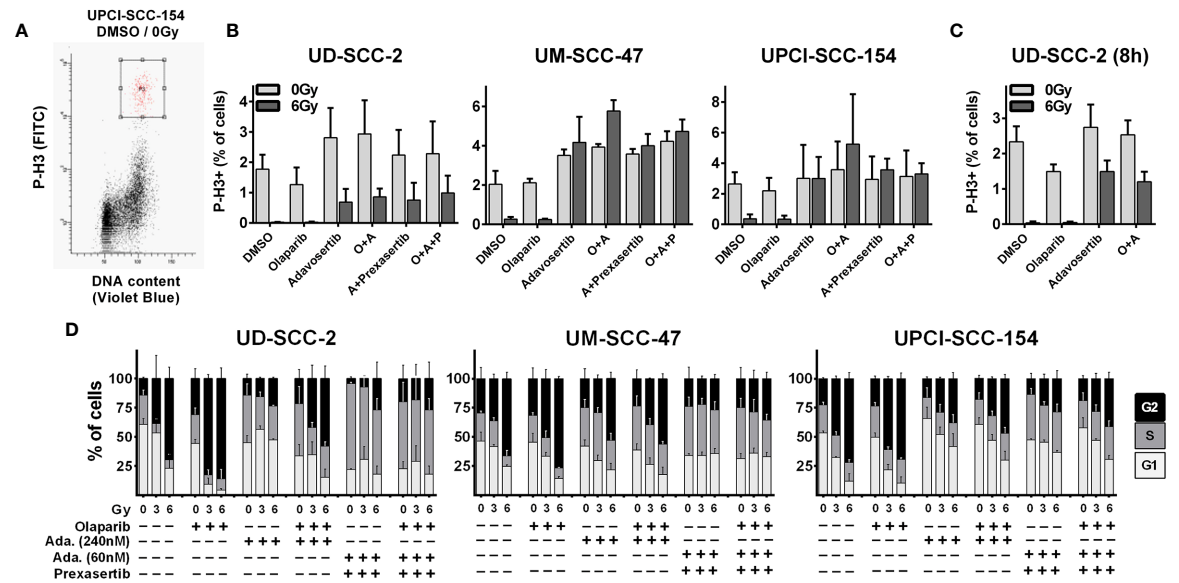

FIGURE 2 | Radiation-induced G2 arrest. **(A–C)** Fraction of mitotic cells. Exponentially growing cells were treated for 2 h with the inhibitors as indicated (olaparib: 1 μM; adavosertib: 240 nM; adavosertib + prexasertib: 60 nm + 1 nM, respectively), before irradiation with 0 or 6 Gy. Five or eight hours after irradiation cells were fixed and stained for phospho-histone H3 (P-H3+) to assess the number of mitotic cells. **(A)** Gating. **(B)** Quantification of the mitotic fraction at 5 h after irradiation. **(C)** Quantification at 8 h after irradiation. **(D)** Long term G2 arrest. Cells were treated and irradiated as in **(A–C)**. Twenty-four hours after irradiation the cells were fixed, and the cell cycle distribution was assessed by DAPI staining and flow cytometry.

As HPV-positive HNSCC cells show prolonged G2-checkpoint responses due to an inefficient DNA DSB repair (33), we further assessed cell cycle distribution at a later time point of 24 h after irradiation where all cell lines demonstrated profound radiation-induced G2 arrest (**Figure 2D**). In line with the short term experiments described above, adavosertib treatment reduced the amount of radiation-induced G2 arrest also at 24 h after irradiation but not to the full extent. The combination of adavosertib and prexasertib also reduced G2 arrest and partly increased the amount of S phase cells, suggesting severe replication stress. Addition of olaparib to adavosertib ± prexasertib did not induce any further accumulation in S-phase irrespective of radiation. In UD-SCC-2 cells, sole olaparib treatment resulted in a clear increase of cells in G2, especially after irradiation but also at baseline. In UM-SCC-47 and UPCI-SCC-154 the increase was subtle but highly reproducible, which is in line with enhanced DNA damage levels after PARP inhibition as frequently reported (**Supplementary Figure S2A**) (42–44). Enhanced damage levels are further supported by higher intensity of the DNA damage marker γH2AX in cells residing in radiation-induced G2-arrest after olaparib treatment in all three cell lines (**Supplementary Figure S2B**).

For all the following experiments, we continued with concentrations of 1 μM olaparib and 240 nM adavosertib or, alternatively, the reduced concentration of 60 nM adavosertib combined with 1 nM prexasertib, which demonstrated similar G2 checkpoint abrogation in these assays.

Replication Stress

Unscheduled activation of dormant origins and subsequent nucleotide depletion is described as a mechanism of antitumor activity through Wee1 and/or Chk1 inhibition (18, 20). This leads to replication stress and, if severe, S-phase arrest as partially observed for the combined Wee1/Chk1 inhibition described above. Chk1 is further described as a replication fork protection factor (45) and PARP1, apart from its functions in DNA repair, was reported to be involved in the restart of stalled replication forks and Chk1-dependent S-phase checkpoint activation and fork protection (46–49).

In S-phase, cell stretches of single-strand DNA (ssDNA) upon replication fork stalling as well as DSBs upon replication fork collapse are recognized through the related ATR and ATM kinases, and such areas are subsequently decorated by γH2AX. In line with these mechanisms, the inhibition of Wee1 as well as the combined inhibition of Wee1/Chk1 resulted in a strong increase in γH2AX signal intensity in S and partly G2 phase cells. However, neither olaparib alone nor the addition of olaparib to Wee1 or to Wee1/Chk1 inhibition resulted in any substantial increase in γH2AX levels with the exception of sole addition in UD-SCC-2 cells. Here, a considerable number of cells demonstrated higher γH2AX levels, but the rise in signal intensity was very modest (**Figures 3A, B** and **Supplementary Figure S3A**). Although less uniform than the γH2AX staining, the results were in principle confirmed when assessing the amounts of chromatin-bound RPA, which, as the primary ssDNA binding and protection factor, represents a very direct and robust marker for replication stress (50) (**Figures 3C, D**). Notably here, in UD-SCC-2 and UPCI-SCC-154, sole Wee1 inhibition resulted in a more moderate induction of RPA signal intensity compared to combined Wee1/Chk1 inhibition, in line with the stronger

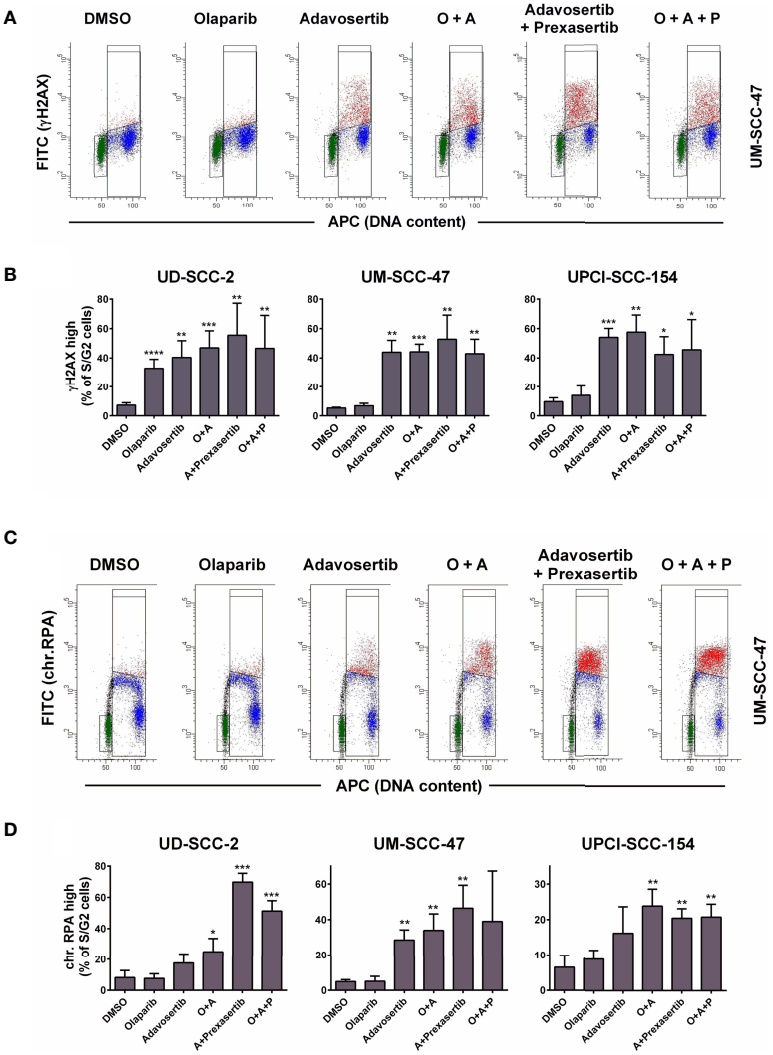

FIGURE 3 | Effect of PARP and intra-S/G2 checkpoint inhibition on γH2AX and chromatin-bound RPA staining intensity. Cells were treated with inhibitors as indicated for 24 h before fixation, staining, and flow cytometric measurements. In case of RPA staining the cells were pre-extracted before fixation. **(A)** Examples of the flow cytometric measurement of γH2AX. Gates are set to select cells in G1 (green), in S/G2 (red & blue) or cells in S/G2 with enhanced γH2AX levels (red). **(B)** Fraction of S/G2 phase cells that demonstrate enhanced γH2AX levels. **(C)** Examples of the flow cytometric measurement of chromatin-bound RPA, which is highest in the replicative S-phase. Gates are set to select cells in G1 (green), in S/G2 (red and blue) or cells in S with enhanced RPA levels (red). **(D)** Fraction of S/G2 phase cells that demonstrate enhanced RPA staining levels. Asterisks depict significant differences to solvent (DMSO) treatment with *, **, *** and **** indicating $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively (two-tailed Student's t-test).

accumulation in the S-phase described above (**Supplementary Figure S3B**). Adding the PARP inhibitor did not prominently change the amount of cells positive for γH2AX or chromatin-bound RPA.

Together these results demonstrate that under Wee1 and especially Wee1/Chk1 inhibition S phase cells will have to repair radiation induced DNA damage under conditions of replication stress and with a severely reduced ability to halt the cell cycle in G2 and therefore without extra time for DNA repair before the critical passage through mitosis. Additional inhibition of PARP did not prominently impact on replication stress or inhibition of G2 arrest according to the endpoints measured.

DSB Repair

The reduced DNA DSB repair capacity of HPV-positive HNSCC cells has been frequently ascribed to a defect in the DNA repair pathway homologous recombination (HR) (28–31) and also a switch towards the error prone alt-EJ pathway has been reported (26, 27). As PARP1 is a key component of the latter (12) and Wee1 has been described as a relevant HR factor (35), we tested the influence of PARP- and Wee1 inhibition on NHEJ and HR using established GFP-based reporter gene constructs stably integrated in HPV-positive UD-SCC-2 and UPCI-SCC-154 cells (51) (**Supplementary Figures S4A, B**). Although the pEJ construct can interrogate classical NHEJ and alt-EJ repair (52),

PARP inhibition did not reduce the rate of measurable NHEJ in either cell line (**Supplementary Figure S4C**). Despite the reported HR defect of HPV-positive HNSCC cells, we had also been able to establish UD-SCC-2 and UPCI-SCC-154 HR reporter cells. Unexpectedly, Wee1 inhibition did not reduce the rate of HR repair as assessed through the pGC reporter construct and the combination with PARP inhibition even increased the rate of GFP-positive cells (**Supplementary Figure S4D**).

In line with the reporter gene assay results, we also did not observe an enhancement of residual 53BP1 nuclear foci as markers of unrepaired DSBs at 24 h after irradiation with 2 Gy under combined PARP/Wee1 inhibition in UD-SCC-2 and UPCI-SCC-154 and only a slight, non-significant increase in UM-SCC-47 (**Figure 4A** and **Supplementary Figure S5A**). We did, however, observe a common phenotype regarding the distribution of foci with respect to the cell cycle phase as determined by geminin co-staining, which marks cells in S and G2 phases (**Figure 4B**). In all cell lines, the average foci number in G1 increased significantly upon combined PARP/Wee1 inhibition, whereas foci in S/G2 phase cells decreased (**Figure 4C** and **Supplementary Figure S5B**). In line with the respective cell cycle data (**Figure 2**), this underscores that under combined inhibition cells with unrepaired DSBs exit G2 arrest and take the critical passage through mitosis despite the enhanced risk of acute and delayed mitotic cell death. In general, cells with low numbers of residual radiation-induced DSBs are the ones most likely to survive and the fraction of such potentially surviving cells after 2 Gy was decreased in all strains upon dual PARP/Wee1 inhibition, albeit in UD-SCC-2 slightly missing significance ($p = 0.0777$) (**Figure 4D**). Regarding cell cycle, this reduction was observed in the G1-phase in all strains, again underpinning premature mitotic passage (**Figure 4E**). Surprisingly, in UD-SCC-2 the fraction of cells with few residual foci was also significantly reduced in S/G2 phase cells upon dual inhibition, despite the overall decrease in average foci numbers in this fraction (**Figure 4C**).

Radiosensitization

So far while we did not observe clear hints pointing towards enhanced cytotoxicity when adding a PARP inhibitor to intra-S/G2 checkpoint inhibition, radiosensitization through PARP inhibition is clearly established owing to an enhanced induction of replication-induced one-ended DSBs, the inhibition of alt-EJ and further mechanisms (53). Moreover, we had previously observed highly effective radiosensitization in HPV-positive HNSCC cells when combining olaparib with the Chk1 inhibitor PF-00477736 (10). In line with these results, a significant reduction of colonies indicating radiosensitization was now observed upon combined PARP/Wee1 inhibition as

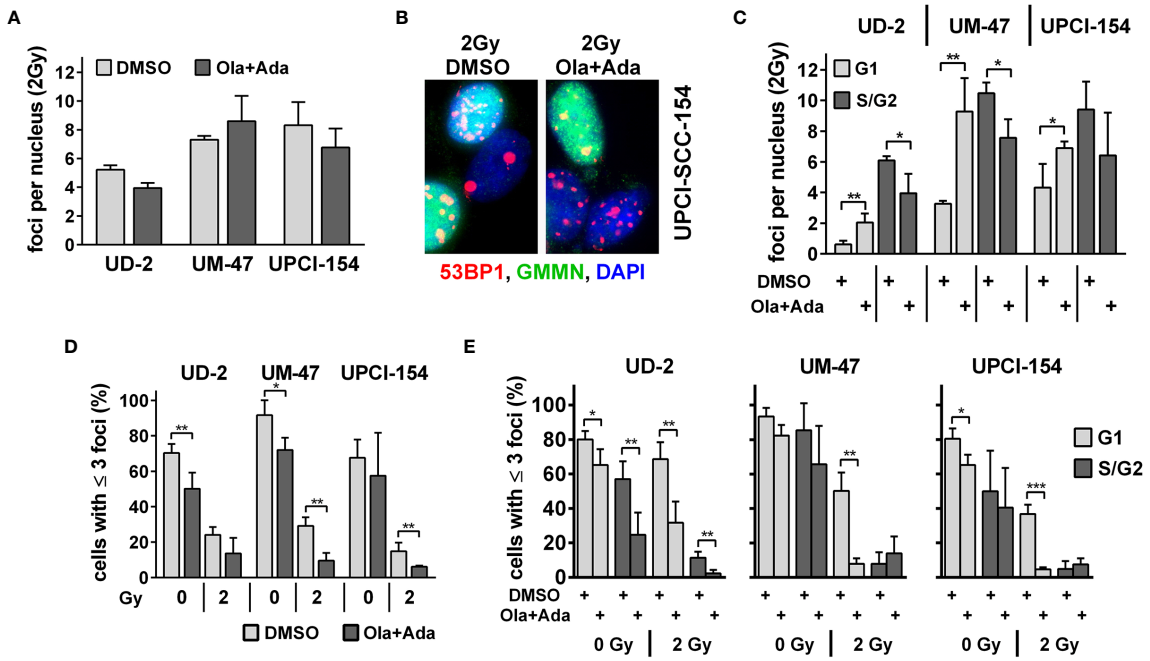

FIGURE 4 | Effect of PARP and Wee1 targeting on DSB repair. **(A)** Quantification of radiation-induced nuclear 53BP1 foci at 24 h after 2 Gy irradiation. Counts were normalized to the DNA content of the respective cell lines as assessed previously (33), foci numbers in non-irradiated controls were subtracted. **(B)** Example of immunofluorescence co-staining of 53BP1 and the S/G2 phase marker geminin (GMMN). **(C)** Quantification of radiation-induced nuclear 53BP1 foci with respect to the cell cycle phase as determined by geminin co-staining. Foci numbers in non-irradiated controls were subtracted. **(D)** Fraction of cells with ≤3 53BP1 nuclear foci. **(E)** Fraction of cells with ≤3 53BP1 nuclear foci with respect to cell cycle phase as determined by geminin co-staining. Significant changes are indicated with *, ** and *** indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (two-tailed Student's t-test).

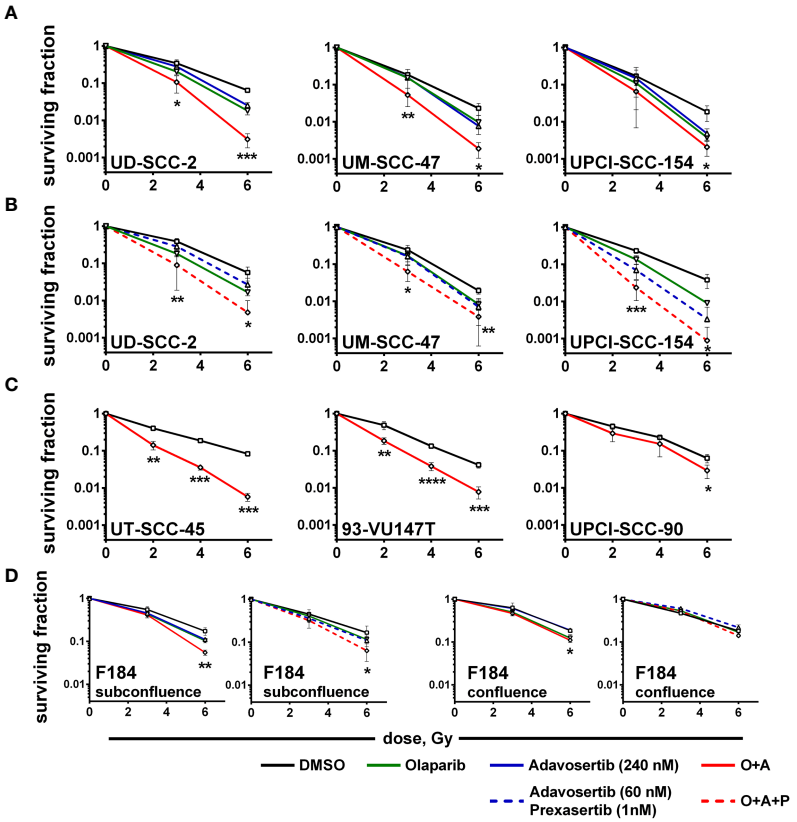

FIGURE 5 | Radiosensitization. Exponentially growing cells were seeded and on the next day treated with inhibitors as indicated and irradiated 2 h thereafter; 24 h later, irradiated cells were seeded in low, defined numbers for colony formation. **(A)** Radiosensitization of HPV-positive HNSCC cells using dual PARP/Wee1 inhibition or **(B)** combined PARP/Wee1/Chk1 inhibition. **(C)** Validation of radiosensitization through combined PARP/Wee1 inhibition using three additional HPV-positive HNSCC cell lines. **(D)** Effect on normal human fibroblasts as an example of normal tissue cells. Significance was assessed for solvent control *vs*. combined PARP + S/G2 checkpoint inhibition. In case of a statistically significant difference the respective dose points are marked with asterisks with *, **, *** and **** indicating $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$, respectively (two-tailed Student's t-test).

compared to single inhibitor usage (**Figure 5A**). Highly similar results were obtained when replacing the 240 nM adavosertib treatment with 60 nM adavosertib/1 nM prexasertib (**Figure 5B**). To further estimate whether radiosensitization occurs in a majority of HPV-positive HNSCC cells, we tested dual PARP/Wee1 targeting in three additional strains, all of which were also sensitized, two very effectively and UPCI-SCC-90 to less extent (**Figure 5C**). To assess tumor specificity, we further tested dual targeting in p53/G1 arrest proficient normal human fibroblasts. In a proliferative state, fibroblasts were radiosensitized by combined inhibition but to a lesser extent than five of the six HPV-positive tumor cell lines. In confluent cultures, the effect of intra-S/G2 checkpoint targeting was completely lost, and radiosensitization was marginal or absent (**Figure 5D** and **Supplementary Table S1**). A comparison of the plating efficiency rates of the non-irradiated controls did not reveal a clear differential effect of the dual *vs.* the triple inhibition approach in HPV-positive HNSCC cells and virtually no reduction of survival in the normal fibroblasts (**Supplementary Figure S6**).

Nucleoside Supplementation Counteracts Radiosensitization Through Wee1 but Not PARP/Wee1 Inhibition

We finally wanted to estimate to what extent the induction of replication stress may contribute to the profound radiosensitization upon combined treatment. As a shortage in nucleotides contributes to replication stress upon intra-S/G2 checkpoint inhibition, it can partly be compensated by external addition of nucleosides (18, 54). To test the effect in our cells, we analyzed γH2AX levels in S-phase cells at 4 h after combined PARP/Wee1 inhibition, a time point corresponding to 2 h post irradiation in the colony formation assays when DSB repair would be highly active. We found γH2AX levels to be induced by combined inhibition in S phase cells and partly suppressed by nucleoside supplementation. A substantial degree of induction and normalization was observed in UD-SCC-2 and UPCI-SCC-154 cells (**Figures 6A, B**). Despite these similarities, nucleoside supplementation did not influence radiation sensitivity in UPCI-SCC-154 but in UD-SCC-2 induced a quite clear trend towards radioresistance in the PARP/Wee1-inhibited samples (6 Gy: $p = 0.0862$). Unexpectedly, resistance was induced in the

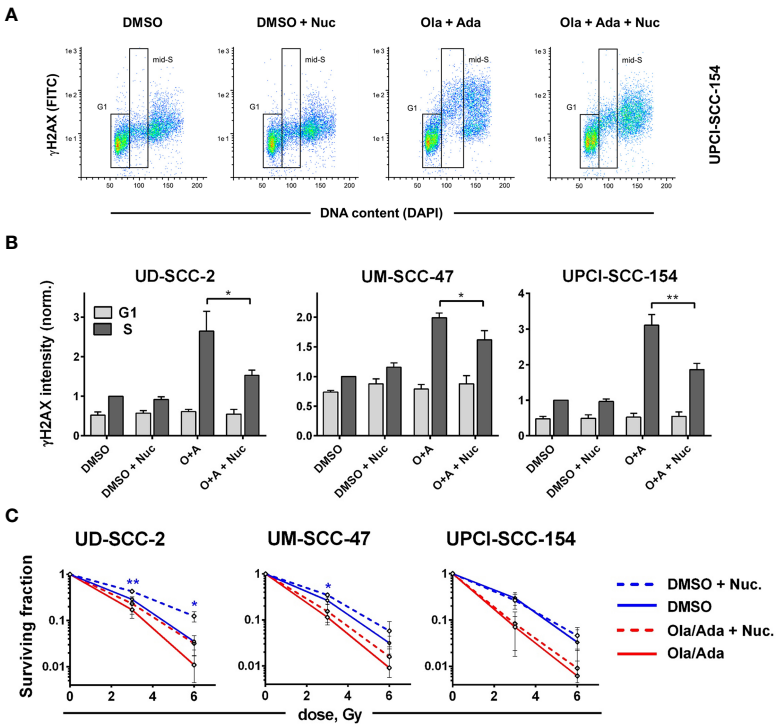

FIGURE 6 | Cell line-dependent induction of radioresistance through nucleoside supplementation. Exponentially growing cells were treated with or without the combination of olaparib and adavosertib and with or without external nucleosides as indicated. **(A)** Example of gating for γH2AX intensity measurement in G1 and mid-S phase cells as assessed by DAPI co-staining. The cells were fixed after 4 h of treatment and analyzed for γH2AX induction by flow cytometry. **(B)** Bars depict the average median γH2AX staining intensity of cells in G1 and mid-S phase. Values were normalized to the intensity of DMSO-treated mid-S phase cells of the respective experiments. Asterisks mark statistically significant differences upon nucleoside supplementation. **(C)** Two hours after addition of inhibitors ± nucleosides the cells were irradiated and after further 24 h seeded for colony formation without addition of inhibitors. Asterisks mark statistically significant differences in survival upon nucleoside supplementation, color indicates solvent controls or inhibitor treatment. Differences between DMSO treatment and dual inhibition without nucleoside supplementation (solid lines) were significant for all cell lines (not indicated). Significant changes are indicated with * and **indicating $p \leq 0.05$ and $p \leq 0.01$, respectively (two-tailed Student's t-test).

solvent-treated controls to a very similar extent reaching significance for the 6 Gy dose point (**Figure 6C**). In comparison, sole Wee1 inhibition induced a similar increase in γH2AX levels, and nucleoside supplementation resulted in a pertinent normalization. In contrast to the situation under combined targeting, nucleoside supplementation counteracted adavosertib-mediated radiosensitization in UM-SCC-47 and UPCI-SCC-154, with no or little effect in the respective solvent-treated controls. Solely in UD-SCC-2, nucleoside supplementation exerted a similar effect on adavosertib and control treated cells (**Supplementary Figure S7**). So while these data strongly suggest that replication stress caused by nucleotide shortage can play a prominent role in the radiosensitization under sole Wee1 inhibition, they question a meaningful role for the radiosensitization under combined PARP/Wee1 inhibition in our cells.

The cause for radioresistance under nucleoside supplementation in solvent treated UD-SCC-2 cells currently remains elusive. In a set of pilot experiments, nucleosides increased the fraction of G1 at the cost of S phase cells in UD-SCC-2 and reduced their proliferation speed (**Supplementary Figures S8A, B**). Also especially in UD-SCC-2, the radiation-induced G2 arrest was diminished upon nucleoside supplementation, suggesting that fewer residual DSBs were present to trigger the G2 cell cycle checkpoint (**Supplementary Figure S8A**). Finally, analyses of residual DSBs under nucleoside supplementation *via* 53BP1 nuclear foci in UD-SCC-2 cells demonstrated an increase in the fraction of cells with few (≤3) foci after irradiation, in line with radioresistance induction. The effect was present and significant in both cells that were or were not actively replicating at the time of irradiation (**Supplementary Figure S8C**). Further analyses will be necessary to clarify this intriguing finding of radioresistance through nucleoside supplementation in otherwise unperturbed cells.

DISCUSSION

Inhibition of Wee1 by adavosertib was recently described as a highly effective single-agent treatment for HPV-positive HNSCC dependent on FOXM1 activation (55) and single agent radio- and chemosensitization through PARP, as well as through intra-S/G2 checkpoint inhibition, which was repeatedly demonstrated in HPV-positive HNSCC models (10, 21–23, 25, 30, 31, 56, 57).

In this study we demonstrate a highly effective radiosensitization of HPV-positive HNSCC cells using dual inhibition of PARP and the S/G2 cell cycle checkpoint in five and moderate radiosensitization in one out of six cell lines tested. A similar result has recently been independently described for the HPV-positive strain UPCI-SCC-154 (24). Here it was suggested that the combination of PARP plus Chk1 inhibition is more effective in HPV-positive HNSCC cells, whereas the combination of PARP plus Wee1 inhibition is more effective in HPV-negative ones but the estimation was based on only one cell line per group. For this particular HPV-positive strain, we have indeed also observed an exceptionally strong radiosensitization when including a Chk1 inhibitor (**Figure 5**). The data are also in line with previous findings of strong radiosensitization using sole Chk1 and combined Chk1/Wee1 inhibition, but again the effect was only specific for UPCI-SCC-154 rather than for HPV-positive cells in general (22). Of note, this strain was also an outlier in the response to the particular Chk1 inhibitor PF-004776, but here demonstrated non-responsiveness for various endpoints, which further suggests irregularities (21). Effective radiosensitization through combined inhibition of PARP and the intra-S/G2 cell cycle checkpoint has also been described for other entities and for different approaches of checkpoint targeting, such as Chk1 or ATR inhibition (10, 58–60). The combination of PARP/Wee1 inhibition was previously tested in lung and pancreatic cancer cells with similarities but also some differences to our findings in HPV-positive HNSCC cells (61, 62). Contrasting these studies we did not observe inhibition of HR upon Wee1 inhibition in plasmid reconstruction assays and we neither observed a reduction of NHEJ upon PARP inhibition despite the reported enhanced usage of alt-EJ in HPV-positive HNSCC (26, 27). Furthermore, while replication stress was clearly evident upon intra-S/G2 checkpoint inhibition, we could not confirm an important role for the radiosensitization under combined inhibition, since for example in UD-SCC-2 targeting the intra-S/G2 checkpoint by combined Wee1/Chk1 inhibition induced replication stress more effectively than sole Wee1 inhibition but radiosensitization was highly similar (**Figures 2**, **3**, **5**). And while external nucleoside supplementation succeeded in partly relieving replication stress, it either failed to reduce radiosensitization (UPCI-SCC-154) or induced radioresistance in the solvent-treated controls to a similar extent as under combined PARP/Wee1 inhibition (UD-SCC-2) (**Figure 6**). In contrast, nucleoside supplementation demonstrated a pertinent reduction in replication stress and effectively counteracted radiosensitization upon sole Wee1 inhibition in two out of three cell lines tested (**Supplementary Figure S7**), suggesting additional mechanisms and a more robust radiosensitization upon combined inhibition. These findings are actually in line with previous reports, where the addition of nucleosides also counteracted radiosensitization under sole Wee1 (62, 63) but not under combined Wee1/PARP inhibition (62). Interestingly, nucleoside supplementation had also induced radioresistance in solvent treated samples in one of three (hepatocellular) carcinoma cell lines tested, while in NSCLC cells no results for the solvent treated controls were presented (62, 63). While clearly not the focus of this manuscript, our observation of profoundly enhanced radioresistance upon nucleoside supplementation in solvent-treated UD-SCC-2 cells is interesting and warrants future mechanistic investigations.

A puzzling finding of our study is the slight reduction in the overall number of 53BP1 foci upon combined treatment (**Figure 4**). In general, an enhancement in DNA damage in S/G2 phase upon PARP inhibition is very well established (13, 14, 64) and, accordingly, we observed an increase in G2 arrested cells and enhanced γH2AX levels in G2 phase cells upon PARP inhibition and moderate radiosensitization here and previously (**Figures 2**, **5** and **Supplementary Figure S2**) (10). A possible explanation, in line with the cell cycle data and the shift in foci number from G2 to G1 phase cells (**Figures 2**, **4**) may be that overriding the otherwise long lasting G2 checkpoint can result in immediate mitotic catastrophe and cell elimination, preferentially of those cells with high damage and foci levels that would otherwise reside long enough in G2 to be scored. In line with this theory, the proportion of irradiated G2 phase cells with ≥20 53BP1 foci decreased in UD-SCC-2 and UPCI-SCC-154 upon combined inhibition (data not shown). Importantly, the fraction of cells with very low foci numbers was reduced upon dual inhibition in all cell lines tested. Overall, our results point towards a mechanism for radiosensitization driven by the abrogation of the, in HPV-positive HNSCC cells extensive, G2 cell cycle arrest in combination with the induction of additional DNA damage in S/G2 through PARP inhibition. While differences may exist in detail, the described effectiveness in different entities and by application of various checkpoint inhibitors clearly point towards a very robust radiosensitization of proliferating tumor cells by this combinatorial approach (37, 38). In contrast, normal fibroblasts, representing p53-proficient normal tissue cells, were only modestly affected in our study (**Figure 5D**), which indicates a fair degree of tumor specificity, especially given that many normal tissues do not or only slowly proliferate.

From the translational view, HPV-positive HNSCC may represent an especially promising entity for radiosensitization through molecular targeting. Patients possess a favorable prognosis and therefore targeting agents may not be added to concomitant chemotherapy (CT) but could rather replace CT and this should reduce, instead of increase, the risk of severe systemic side effects. Safe de-intensification of treatment is already the common goal in clinical trials for HPV-positive HNSCC. A major drawback, however, was the reported inferiority of cetuximab compared to cisplatin despite maintaining full dose radiotherapy in two phase 3 trials (65). These studies clearly highlight the need for effectiveness and thorough preclinical evaluation of molecular targeting approaches despite the overall favorable prognosis. In the frame of recent clinical data on de-intensification, promising initial results were obtained for reducing radiation dose in definitive chemoradiation and after induction chemotherapy (ICT) (3, 5, 7). In the frame of the latter, effective targeting may also be an alternative to adjuvant chemotherapy after ICT in the frame of risk-adapted, de-intensified radiotherapy and may evade potential chemoresistance mechanisms selected for or acquired during ICT. All inhibitors used in this study are

already being tested in clinical trials in combination with radiotherapy in HNSCC (66, 67) (NCT02555644, NCT01758731, NCT02308072, NCT02585973). Olaparib is clinically approved in other entities, and the combination of adavosertib and radiotherapy (plus gemcitabine) was recently reported to yield promising results in pancreatic cancer (68). Moreover, combined treatment with olaparib and adavosertib as well as with prexasertib is also being clinically tested in a number of entities (NCT02576444, NCT02511795, NCT03579316, NCT03330847), albeit so far not in combination with radiotherapy. From our point of view, the clinical stage of the inhibitors available and the preclinical evidence provided in this study clearly warrant subsequent *in vivo* experiments as a next step towards a possible clinical exploration of the described approaches in the frame of de-intensification trials in HPV-positive HNSCC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KH, TB, AO, SC, and FG conducted experiments under the supervision of TR. KH, TB, AO, SC, FG, and TR analyzed the data. MK, KR, CP, CB, NS and TR contributed conception and design of the study; AO, NS, KR, and TR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.683688/full#supplementary-material

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Choosing the Right Treatment Option for the Right R/M HNSCC Patient: Should We Adhere to PFE for First-Line Therapy?

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Background: The landmark EXTREME trial established cisplatin, 5-fluorouracil and cetuximab (PFE) as first-line chemotherapy (1L-ChT) for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). We were interested in outcome differences of R/M HNSCC in 1L-ChT and factors influencing outcome in certain subgroups, especially patients receiving PFE, and the value of PFE compared to other 1L-ChT regimens to provide real world evidence (RWE).

Methods: For this retrospective monocentric study, 124 R/M HNSCC patients without curative surgical or radiotherapy options receiving at least one cycle of 1L-ChT were eligible. We analyzed their outcome using Kaplan-Meier plot and Cox regression to identify predictors for prolonged survival.

Results: Subgroups benefiting significantly from PFE were patients suffering from an index HNSCC outside the oropharynx. The PFE regimen proved to be superior to all other 1L-ChT regimens in clinical routine. Significant outcome differences between PFE treatment within or outside controlled trials were not seen.

Conclusion: This retrospective analysis provides RWE for factors linked to improved outcome. Subgroup analyses highlight the lasting value of PFE among the growing spectrum of 1L-ChT. Importantly, fit smokers with high level alcohol consumption benefit from PFE; considering the patient's lifestyle factors, PFE should not be ignored in decision-making.

Keywords: head and neck cancer, head and neck squamous cell carcinoma, palliative chemotherapy, first-line therapy, recurrent/metastatic head and neck squamous cell carcinoma, multivariate Cox proportional hazard regression, outcome research, p16+ oropharyngeal cancer

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INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC) is an entity with growing importance, in clinical but also in research settings. According to the EUROCARE-5 trial (1), there were 238,608 cases recorded from 1999 to 2007 in Europe. Five-years overall survival (OS) for all HNSCC entities was 42.2% (95% confidence interval, 95%CI: 41.5–42.9%), ranging from 25.5% for oropharynx to

61.1% for larynx cancer. At initial diagnosis of HNSCC, 54.0% of all HNSCC were classified as UICC IV due to regional or distant metastasis. According to the NCCN Guidelines for Head and Neck Cancer (2018) (2), curative therapy is considered appropriate until UICC IVB, whereas detection of distant metastasis (M1 defining stage IVC) means loss of curative treatment options advising switch to systemic treatment and palliative care (with the only exception of resectable solitary M1). The same applies to recurrent locally advanced HNSCC after radiotherapy without resectability. While there are certain therapy algorithms for HNSCC in curable stages, only a few approved options for first-line chemotherapy and other systemic first-line therapies (altogether summarized under the abbreviation 1L-ChT) are available in case of R/M HNSCC following the NCCN guidelines from 2018. Since publication of the landmark EXTREME trial (3), treatment with up to six cycles of cisplatin, 5-fluorouracil and cetuximab (PFE), became standard 1L-ChT in R/M HNSCC. After the KEYNOTE-048 trial (4), this standard was recommended being replaced by a stratified 1L-ChT according to programmed death ligand 1 (PD-L1) expression assessed by combined positivity score (CPS). According to immune histopathology, PFE remains a standard of care for patients with a CPS <1, whereas patients with a CPS ≥20 should be treated with pembrolizumab mono and patients with CPS ≥1 and <20 should receive cisplatin/5-fluoruracil/pembrolizumab (5).

Prior trials often used PFE as control arm (6–9), but new 1L-ChT options superior to PFE have not yet been identified or been established based on lower toxicity. In the course of precision medicine and decision-making for stratified therapy regimens leading to a more individualized or even personalized treatment, new therapy options became eligible for specific groups of patients as second-line therapy (2L-ChT) or 1L-ChT for patients not eligible for PFE (frail patients and/or insufficient kidney or liver function). We were interested in the outcome of PFE *versus* the other 1L-ChT and predictors for good outcome after PFE therapy and consequentially aimed on defining groups of patients that still benefit the most from PFE as part of a widened spectrum of therapy options.

MATERIALS AND METHODS

Study Population and Patient Samples

Eligible for the study were patients with pathological confirmed R/M HNSCC treated in the University Hospital Leipzig with data recorded in the Microsoft Access® tumor database (TDB) of the ENT department, comprising data of all patients diagnosed with a malignant disease since 1990, and data taken from the hospital's electronic health records. **Figure 1** shows the selection of patients for analyses according to the CONSORT recommendations. Among 346 R/M HNSCC patients presented to the multidisciplinary tumor board (MDTB; see below), 130 R/M HNSCC without curative treatment option were subjected to systemic therapy and received at least one cycle 1L-ChT. To prevent any inconsistency based on minor R/M HNSCC subgroups, patients with primary HNSCC localized in the nasopharynx (ICD10-C11), or nasal cavity (ICD-10-C30 and C31) were excluded from the present analyses resulting in a sample of 124 patients (**Table 1**). Pathological reports were available for all 124 patients. All resected specimen underwent pathological examination, and hematoxylin–eosin (HE) staining revealed squamous cell carcinoma histology. A sub-cohort of patients participated in a study approved by our Ethics Committee (votes 201-10-12072010 and 202-10-12072010).

Clinical Work-Up for R/M HNSCC

As recommended (2), clinical work-up for R/M HNSCC included clinical examination, ultrasound sonography, contrast-enhanced CT for head and neck and thorax, eventually PET-CT/PET-MRI, followed by a panendoscopy accompanied by taking biopsies before decision-making for treatment in the MDTB. Patient and tumor characteristics, diagnostic procedures, treatment and clinical follow-up were recorded in our Microsoft Access® tumor database (TDB) and OncoFlow® (10, 11).

CT and PET-CT Imaging

According to clinical guidelines, all patients received a head and neck and a CT scan of the chest during staging. In 2006, a PET-CT became available. An experienced board-certified nuclear-medicine physician and a radiologist analyzed PET-CTs. Sites of tumor involvement were identified visually by enhanced, non-physiologically [^{18}F]-FDG uptake.

Decision-Making Process in the MDTB

The decision-making process in the MDTB followed ASCO and NCCN guidelines (2) and principles published earlier (10–13). Briefly, a radiologist and a nuclear medicine specialist presented all radiological imaging. The MDTB consisting of head and neck and maxillofacial surgeons, a pathologist, an oncologist, a radiation oncologist, and other clinical staff involved in the treatment of head and neck cancer patients discussed the results of diagnostic procedures. Considering the general health and comorbidity of the patient the pre-therapeutic MDTB regarding the guidelines (2) recommended the type of 1L-ChT according to inclusion criteria of open randomized controlled trials (RCTs) or according to fitness for current therapy standards, PFE or other 1L-ChT. For the subgroup of patients receiving 1L-ChT other than PFE, the most relevant RCTs were CeFCiD (NCT02268695), RESGEX (NCT02052960) and ADVANTAGE (NCT00705016) (6–8).

Immunohistochemistry for P16 and HPV Genotyping

Before decision-making in MDTB and starting therapy, biopsies were taken under general anesthesia and underwent pathological examination. Pathological reports were available for all 124 patients. Besides hematoxylin–eosin (HE) staining, molecular analyses of p16 by immunohistochemistry utilizing the CINtec kit (Roche) were done in oropharynx squamous cell carcinomas (OPSCC) of RCT participants and performed in OPSCC routinely since 2013. Double-stained, p16-positive/Ki67-

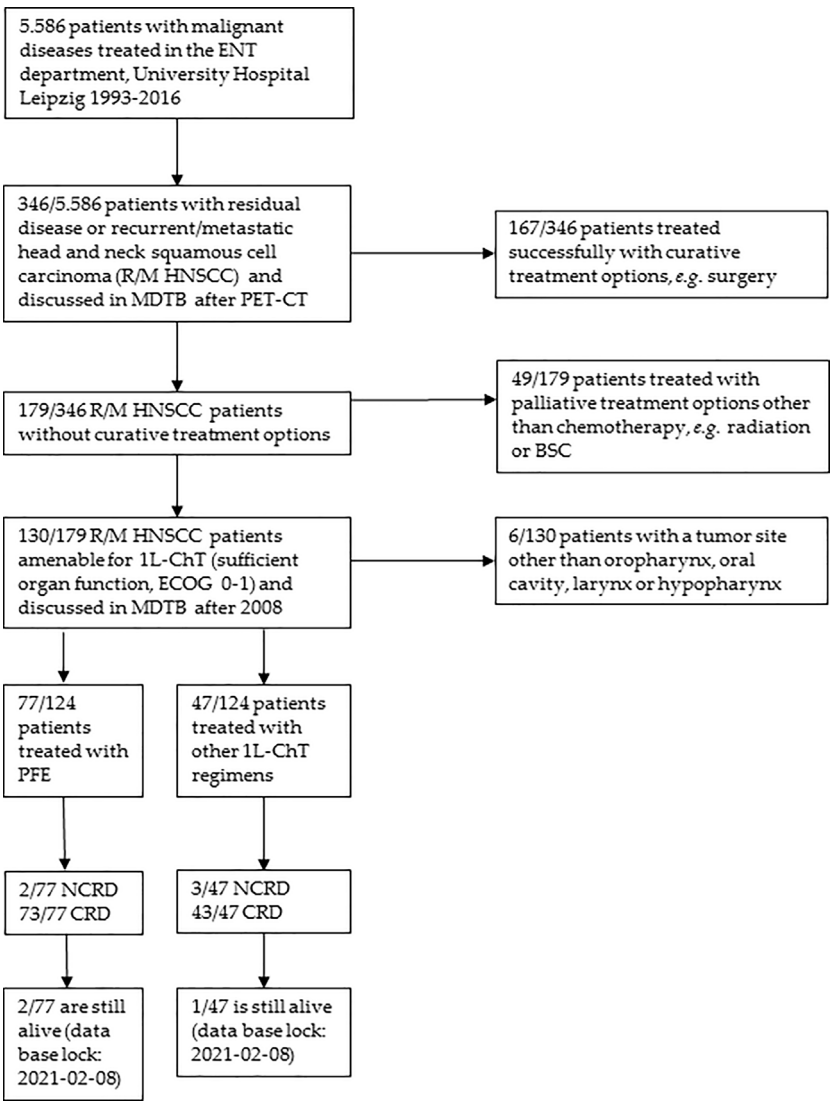

FIGURE 1 | CONSORT diagram showing the selection criteria of recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) patients of the two cohorts compared. ECOG, Eastern Cooperative Oncology Group performance score; BSC, Best Supportive Care; MDTB, multi-disciplinary tumor board; NCRD, non cancer-related death; CRD, cancer-related death.

positive cells or a cutoff level of ≥70% p16-positive OPSCC cells were considered p16+. DNA of p16+ OPSCC was extracted and analyzed for high-risk human papillomavirus utilizing the INNO-LiPA HPV Genotyping Extra kit (Innogenetics) as described earlier (14).

Statistical Analysis

Overall survival (OS) was the time from initial diagnosis of HNSCC to cancer-related (CRD) or non-cancer-related death (NCRD) censoring patients alive at the end of follow-up (data base lock: 08.02.2021). Survival after 1L-ChT ($OS_{1L\text{-}ChT}$) was the time from diagnosis that led to 1L-ChT until death by any cause, censoring patients alive at the end of follow-up or data base lock. We performed a statistical analysis in SPSS 25®. We used *Chi-square* tests, paired and heteroscedastic *t*-tests, receiver-operating characteristics (ROC) curves and Fisher's exact test to investigate the association of clinical characteristics and the outcome of patients receiving PFE or other 1L-ChT. Kaplan–Meier cumulative survival plots and log-rank tests were used to investigate the impact of particular characteristics on $OS_{1L\text{-}ChT}$. We analyzed all parameters achieving P <0.2 in univariate models in multivariate Cox proportional hazard regression (MCR) models. After checking collinearity, independent predictors for the $OS_{1L\text{-}ChT}$ have been identified in MCR applying the step-wise-forward method. For internal validation and to reduce over-optimism based on the effects of random sampling errors, we utilized bootstrapping (1,000 iterations). We considered P <0.05 being significant.

TABLE 1 | Clinical and epidemiological characteristics, diagnostic procedures and treatment as well as various survival measures for 5-years outcome of recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) patients of the two subgroups, PFE and other 1L-ChT.

Characteristics		**Number of Patients (%)**						
		Total	***N* = 124**	**PFE**	***N* = 77**	**other 1L-ChT**	***N* = 47**	***P*-value***
Age at inital diagnosis, years	<50	29	(23.4)	16	(20.8)	13	(27.7)	0.272
	50–60	48	(38.7)	35	(45.5)	13	(27.7)	
	60–70	40	(32.3)	22	(28.6)	18	(38.3)	
	>70	7	(5.6)	4	(5.2)	3	(6.4)	
	median (IQR)	56.7	(50.2-63.7)	56.6	(50.2–63.1)	58.4	(49.4–64.1)	0.592
Age at 1L-ChT, years	<50	19	(15.3)	11	(14.3)	8	(17.0)	0.199
	50–59	50	(40.3)	36	(46.8)	14	(29.8)	
	60–69	45	(36.3)	26	(33.8)	19	(40.4)	
	>70	10	(8.1)	4	(5.2)	6	(12.8)	
	median (IQR)	58.8	(53.2–65.6)	58.1	(53.1–65.5)	61.0	(54.1–66.4)	0.279
ECOG	0–1	123	(99.2)	77	(100.0)	46	(97.9)	0.199
	2	1	(0.8)	0		1	(2.1)	
Sex	Male	105	(84.7)	67	(63.8)	38	(80.9)	0.355
	Female	19	(15.3)	10	(13.0)	9	(19.1)	
Alcohol, status	Missing	7	(5.6)	5	(6.5)	2	(4.3)	0.991
	Never	15	(12.1)	9	(11.7)	6	(12.8)	
	Former	13	(10.5)	8	(10.4)	5	(10.6)	
	Current	89	(71.8)	55	(71.4)	34	(72.3)	
Alcohol, (g/d)	Missing	7	(5.6)	5	(6.5)	2	(4.3)	0.999
	0 g/d	15	(12.8)	9	(11.7)	6	(12.8)	
	<30 g/d	36	(30.8)	22	(28.6)	14	(29.8)	
	30–60 g/d	29	(24.8)	18	(23.4)	11	(23.4)	
	>60 g/d	37	(31.6)	23	(29.9)	14	(29.8)	
Tobacco smoking, status	Missing	5	(4.0)	4	(5.2)	1	(2.1)	0.490
	Never	13	(10.5)	6	(7.8)	7	(14.9)	
	Former	24	(19.4)	15	(19.5)	9	(19.1)	
	Current	82	(66.1)	52	(67.5)	30	(63.8)	
Tobacco smoking history, pack years	Missing	7	(5.6)	4	(5.2)	3	(6.4)	0.489
	<30 py	59	(47.6)	35	(45.5)	24	(51.1)	
	>30 py	58	(46.8)	38	(49.4)	20	(42.6)	
Localization	L-/HPSCC	31	(25.0)	17	(22.1)	14	(29.8)	0.053
	OSCC	45	(36.3)	23	(29.9)	22	(46.8)	
	OPSCC	42	(33.9)	32	(41.6)	10	(21.3)	
	other	6	(4.6)	5	(6.5)	1	(2.1)	
p16 status	p16 positive	17	(13.7)	13	(16.9)	4	(8.5)	0.188
	p16 negative	107	(86.3)	64	(83.1)	43	(91.5)	
HPV status	HPV positive	15	(12.1)	12	(15.6)	3	(6.4)	0.127
	HPV negative	109	(87.9)	65	(84.4)	44	(93.6)	
Initial UICC	Missing	1	(0.8)	1	(1.3)		–	0.227
	I	14	(11.3)	7	(9.1)	7	(14.9)	
	II	11	(8.9)	4	(5.2)	7	(14.9)	
	III	12	(9.7)	6	(7.8)	6	(12.8)	
	IVA	52	(41.9)	33	(42.9)	19	(40.4)	
	IVB	15	(12.1)	12	(15.6)	3	(6.4)	
	IVC	19	(15.3)	14	(18.2)	5	(10.6)	
Duration of disease, months	median (IQR)	15.1	(7.2–33.1)	10.7	(6.4–30.4)	21.8	(9.8–37.3)	0.186
Extent of disease	LRR	39	(31.5)	19	(24.7)	20	(42.6)	**0.038**
	M1	85	(68.5)	58	(75.3)	27	(57.4)	
Previous treatments	None	10	(8.1)	9	(11.7)	1	(2.1)	0.104
	One	66	(53.2)	44	(57.1)	22	(46.8)	
	Two	40	(32.3)	19	(24.7)	21	(44.7)	
	Three	6	(4.8)	4	(5.2)	2	(4.3)	
	Four	2	(1.6)	1	(1.3)	1	(2.1)	
Type of prior treatment	No prior ChT	56	(45.2)	36	(46.8)	20	(42.6)	0.652
	• none	*10*	*(17.9)*	*9*	*(25.0)*	*1*	*(5.0)*	
	• PORT	*28*	*(50.0)*	*20*	*(55.6)*	*8*	*(40.0)*	
	• RT	*8*	*(14.3)*	*4*	*(11.1)*	*4*	*(20.0)*	

(Continued)

TABLE 1 | Continued

Characteristics		Number of Patients (%)						
		Total	***N* = 124**	**PFE**	***N* = 77**	**other 1L-ChT**	***N* = 47**	***P*-value***
	• OP	*10*	*(17.9)*	*3*	*(8.3)*	*7*	*(35.0)*	
	Prior ChT	68	(54.8)	41	(53.2)	27	(57.4)	
	• CRT	*14*	*(20.6)*	*9*	*(22.0)*	*5*	*(18.5)*	
	• PORCT	*54*	*(79.4)*	*32*	*(78.0)*	*22*	*(81.5)*	
RCT enrollment	No	56	(45.2)	43	(55.8)	13	(27.7)	**0.002**
	Yes	68	(54.8)	34	(44.2)	34	(72.3)	
	• 1L trial	*68*	*(100.0)*	*34*	*(50.0)*	*34*	*(50.0)*	
	• 2L trial	*13*	*(19.1)*	*11*	*(32.4)*	*2*	*(5.9)*	
Prior cisplatin	Yes	61	(49.2)	36	(46.8)	25	(53.2)	0.487
	No	63	(50.8)	41	(53.2)	22	(46.8)	
Further	no	97	(78.2)	56	(72.7)	41	(87.2)	0.058
therapies	2L-/3L-ChT	27	(21.8)	21	(27.3)	6	(12.8)	
OS status	alive	3	(2.4)	2	(2.6)	1	(2.1)	0.577
	NCRD	5	(4.0)	2	(2.6)	3	(6.4)	
	CRD	116	(93.5)	73	(94.8)	43	(91.5)	

**P value from Pearson's Chi-square (χ^2) tests. PFE, Cisplatin/5-fluoruracil/cetuximab—EXTREME regimen; IQR, Interquartile range; 1L-ChT, first-line chemotherapy; py, pack years; L-/HPSCC, laryngeal/hypopharyngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma; OPSCC, oropharyngeal squamous cell carcinoma; UICC, tumor stages according to the Union International Contre le Cancer; LRR, locoregional recurrence; M1, distant metastasis; ChT, chemotherapy; PORT, postoperative radiation; RT, primary radiation; OP, surgical therapy; CRT, combined chemo-radio-therapy; PORCT, postoperative chemo-radio-therapy; RCT, randomized controlled trial; 1L trial, first-line controlled trial; 2L trial, second-line controlled trial; 2L-/3L-ChT, second-/third-line chemotherapy; OS, Overall Survival; NCRD, Non-cancer-related death; CRD, cancer-related death.*
P values from Pearson's Chi-square tests < 0.05 are in bold.

RESULTS

Patients' Characteristics

Of 124 R/M HNSCC patients, 77 received PFE (**Table 1**). The frequency of PFE was numerically higher in patients younger than 60 years (68.1% *vs.* 54.5%; X^2 = 2.4, P = 0.122). Other 1L-ChT regimens applied to 47 patients not receiving PFE were PFE plus docetaxel (TPFE; n = 15) according to the CeFCiD trial (6) and other cisplatin-based regimens (n = 21 in total, every subgroup n <5); 11/47 patients received in 1L-ChT docetaxel plus cetuximab (n = 3) or a monotherapy with methotrexate (n = 1) or immunotherapy with either cetuximab (n = 3) or nivolumab (n = 4). ECOG performance status in subgroups receiving PFE or other 1L-ChT did not differ significantly (**Table 1**).

Patients' Clinical Course Before and After 1L-ChT

The median time from the initial diagnosis of HNSCC to 1L-ChT was 15.1 months for the total cohort. There was no significant correlation between the time to 1L-ChT and the lifestyle-associated risk factors or patients' age. Patients receiving surgery followed by postoperative radio-chemotherapy (PORCT; n = 52) had a prolonged median time from curative treatment to 1L-ChT of 30.6 months (95%CI: 21.5–40.2) compared to 10.4 months (95% CI: 0.3–20.4) of patients with other types of curative treatment (radiation, surgery, surgery followed by postoperative radiation; n = 62). Median time from initial diagnosis of HNSCC to death/lost to follow-up (OS) was 25.5 months; median time from start of 1L-ChT to death/lost to follow up ($mOS_{1L\text{-}ChT}$) was 8.4 months; 21/124 (16.9%) died within 3 months after starting 1L-ChT (14.3% after PFE, 21.3% after other 1L-ChT regimen). Of 124 patients progressing after 1L-ChT, 27/124 (21.8%) were fit enough to receive either a 2L-ChT or further therapies, 21/77 (27.3%) after PFE, 6/47 (12.8%) after other cisplatin-based regimen. None of the patients treated without cisplatin-based 1L-ChT including all 1L-immunotherapies were fit enough for any 2L-ChT.

$OS_{1L\text{-}ChT}$ After PFE Compared to Other 1L-ChT Regimen

In Kaplan–Meier plots utilizing log-rank tests, a difference of 3 months in $mOS_{1L\text{-}ChT}$ was identified between patients being treated with PFE and those being treated with other 1L-ChT ($mOS_{1L\text{-}ChT}$ (95%CI): 9.8 months (8.1–11.5) *vs.* 6.8 months (3.9–9.7); P = 0.066; **Figure 2A**).

$OS_{1L\text{-}ChT}$ after PFE in RCT *Versus* Clinical Routine

In the group of patients not enrolled in first-line RCT ("real world patients"), a significant benefit from PFE was noticed [$mOS_{1L\text{-}ChT}$ (95%CI): 9.3 (3.3–15.3) months *vs.* 4.1 (1.8–6.4) months, P = 0.016; **Figures 2B** and **4**]. In RCT, other 1L-ChT combined *vs.* PFE showed a similar OS [$mOS_{1L\text{-}ChT}$ (95%CI): 7.0 (3.0–11.0) months *vs.* 9.8 (8.7–11.9) months; P = 0.701; **Figure 2C**]. $OS_{1L\text{-}ChT}$ after PFE outside controlled trials [$mOS_{1L\text{-}ChT}$ (95%CI): 9.3 (3.3–11.3) months) was not significantly different from $mOS_{1L\text{-}ChT}$ in RCTs (9.8 (8.7–10.9) months; P = 0.728; **Figure 2D**]. Of seven long-term survivors within the subgroup of patients treated with PFE in clinical routine (**Figure 2D**), 4/7 were current drinkers, only 1/7 drank >30 g/d alcohol, and 5/7 were current smokers. The median age (56.9 years) was comparable to the median age in the total PFE subgroup (56.6 years, **Table 1**). Five of them (71.4%) had been treated with cisplatin prior to PFE, compared to 46.8% in the total PFE cohort (**Table 1**).

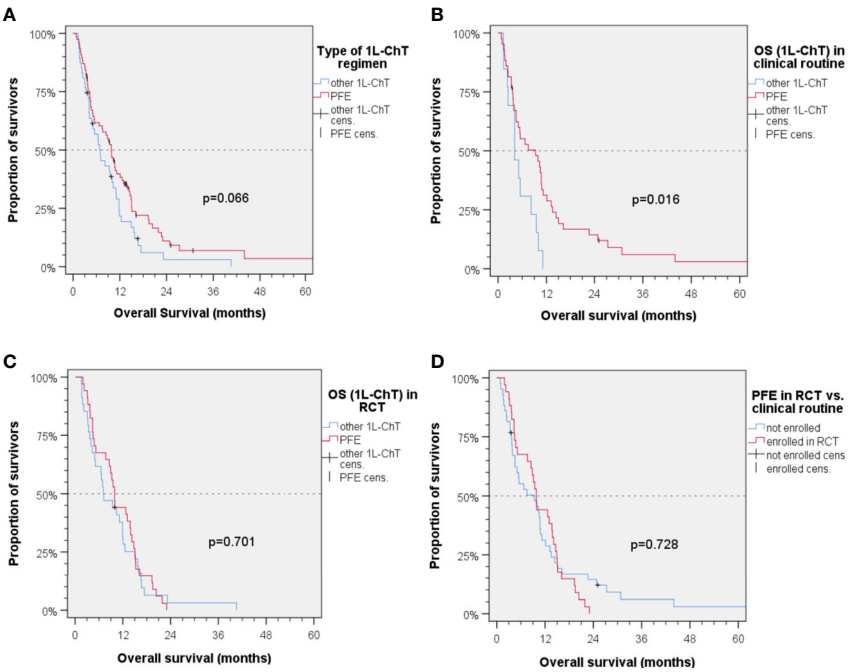

FIGURE 2 | Kaplan–Meier plots for cumulative overall survival ($OS_{1L\text{-}ChT}$) measured from diagnosis of incurable recurrent/metastatic head and neck squamous cell carcinoma receiving first-line chemotherapy as indicated; **(A)** $OS_{1L\text{-}ChT}$ after PFE according to the EXTREME protocol *vs*. other 1L-ChT regimens; **(B)** $OS_{1L\text{-}ChT}$ of patients receiving outside randomized controlled trials (RCT) PFE *vs*. other 1L-ChT regimens; **(C)** $OS_{1L\text{-}ChT}$ of patients receiving PFE *vs*. other 1L-ChT within RCT; **(D)** $OS_{1L\text{-}ChT}$ of patients receiving PFE in RCT *vs*. outside RCT; *P* values shown are from 2-sided log-rank tests.

$OS_{1L\text{-}ChT}$ After Other 1L-ChT Regimen

In this subgroup, the enrollment in RCT was predictive for improved $OS_{1L\text{-}ChT}$ only in these 34 *vs.* 13 patients ($mOS_{1L\text{-}ChT}$ (95%CI): 9.3 (4.7–13.9) *vs.* 4.1 (1.8–6.4); $P = 0.013$). The small number of patients with other 1L-ChT ($n = 47$), however, did not allow to identify further predictors for $OS_{1L\text{-}ChT}$ in this subgroup.

Predictive Factors for $OS_{1L\text{-}ChT}$ After PFE

Kaplan–Meier plots showed the number of pretreatments to be important for therapy outcome in general. Patients initially diagnosed in the metastatic or very advanced stage or after two or more pretreatments had significantly shorter $OS_{1L\text{-}ChT}$ than those receiving 1L-ChT after one pretreatment ($mOS_{1L\text{-}ChT}$ (95% CI): 6.8 (4.2–9.4) *vs.* 9.9 (7.6–12.2) months; $P = 0.038$). Stratified by PFE *vs*. other 1L-ChT, there was still a statistical trend for this finding (**Figure 4**). Patients progressing after cisplatin-based ChT treated with PFE 1L-ChT had prolonged $mOS_{1L\text{-}ChT}$ (9.9 *vs.* 6.8 months; $P = 0.082$; **Figure 4**). Cisplatin-based ChT as part of multimodal pretreatment in the curative setting was equally predictive for $OS_{1L\text{-}ChT}$ in univariate Cox regression model.

Kaplan–Meier analysis linked outcome and age: the $mOS_{1L\text{-}ChT}$ in the age groups (a) ≤49 years (7.6 months, 95%CI: 0.2–15.0), (b) 50–59 years (9.3 months, 95%CI: 7.6–11.0), and (c) ≥60 years (6.8 months, 95%CI: 4.6–9.0) was insignificantly different ($P = 0.192$). Stratified by PFE *vs.* other 1L-ChT, the statistical trend proved to be true and revealed patients aged 50–59 years having the longest $OS_{1L\text{-}ChT}$ independent from the type of 1L-ChT applied ($mOS_{1L\text{-}ChT}$ (95%CI): 9.8 (7.7–11.9) after PFE *vs.* 8.2 (0.0–17.6) months after other 1L-ChT regimen; $P = 0.560$). There were only 11 *vs.* 8 patients aged ≤ 49 years, the $mOS_{1L\text{-}ChT}$ after PFE *vs.* other 1L-ChT was 10.3 (95%CI: 1.6–19.0) months *vs.* 3.3 (95%CI: 0.0–8.0) months (Δ 7.0 months; $P = 0.754$). However, there was a statistical trend in patients ≥ 60 years (30 *vs.* 25 patients) for improved $mOS_{1L\text{-}ChT}$ after PFE *vs.* other 1L-ChT of 7.5 (95%CI: 1.6–13.4) months *vs.* 6.4 (95%CI: 3.6–9.2) months (Δ 1.1 months; $P = 0.082$; **Figure 4**). Among PFE-treated patients, we did not see an inferior $OS_{1L\text{-}ChT}$ of patients older than 65 years compared to younger patients (21 *vs.* 56 patients; $OS_{1L\text{-}ChT}$ (95%CI): 9.9 (1.3–18.5) *vs.* 9.3 (6.9–11.7); $P = 0.467$). Even with a slightly different cut-off point of 60 years (30 *vs.* 47 patients then), we did not see a significant difference neither ($OS_{1L\text{-}ChT}$ (95% CI): 7.5 (1.6–13.4) *vs.* 9.9 (8.0–11.8); $P = 0.974$). However, the heterogeneity in response to PFE in older patients is demonstrated by the enlarged 95%CI.

Regarding different localizations of the primary site of the R/M HNSCC, a statistical trend for oropharyngeal cancer *vs.* HNSCC outside oropharynx was found in Kaplan–Maier analyses ($mOS_{1L\text{-}ChT}$ (95%CI): 6.8 (2.9–10.7) months *vs.* 9.5 (6.6–12.4) months; $P = 0.281$; **Figure 3A**). Analyzing the PFE subgroup (n = 77), this difference was more than 3 months ($OS_{1L\text{-}ChT}$ (95%CI): 7.6 (2.5–12.7) *vs.* 10.7 (9.2–12-2); $P = 0.097$, **Figure 3B**). The p16-status was critical for $OS_{1L\text{-}ChT}$. As p16-positive (p16+) OPSCC had $mOS_{1L\text{-}ChT}$ of 9.3 (95%CI: 4.6–14.0) months comparable with non-oropharyngeal cancer (9.5 (6.6–12.4) months; $P = 0.784$), p16-negative OPSCC had impaired

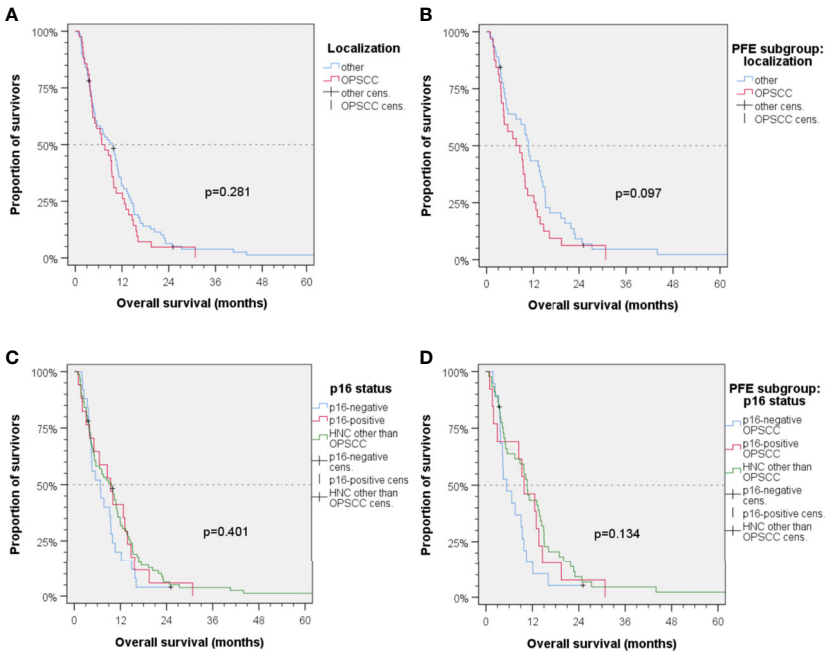

FIGURE 3 | Kaplan–Meier plots for cumulative overall survival ($OS_{1L\text{-}ChT}$) measured from diagnosis of incurable recurrent/metastatic head and neck squamous cell carcinoma **(A, C)** in the total cohort and **(B, D)** PFE treated patients. **(A)** $OS_{1L\text{-}ChT}$ for OPSCC *vs.* index HNSCC outside the oropharynx; **(B)** $OS_{1L\text{-}ChT}$ after PFE for OPSCC *vs*. HNSCC outside the oropharynx; **(C)** $OS_{1L\text{-}ChT}$ in p16-negative OPSCC *vs*. p16-positive OPSCC *vs*. HNSCC outside the oropharynx; **(D)** $OS_{1L\text{-}ChT}$ after PFE in p16-negative *vs*. p16-positive *vs*. HNSCC outside the oropharynx; *P* values shown are from 2-sided log-rank tests.

$mOS_{1L\text{-}ChT}$ of 6.7 (95%CI: 2.9–10.5) months (**Figure 3C**). Stratified by type of 1L-ChT, we saw impaired $OS_{1L\text{-}ChT}$ in p16-negative OPSCC patients even if PFE treated (**Figure 3D**). Considering HPV-driven OPSCC (n = 15 p16+ HR-HPV-DNA+ OPSCC out of n = 17 p16+ OPSCC) did not result in deviating measures but reduced differences due to enlarged 95% CI and increased *P* values, besides use of sole p16-IHC in clinical routine the reason for reporting results for p16+ OPSCC in **Figures 3–5**.

Patients with index HNSCC outside the oropharynx had a significant benefit from PFE *vs.* other 1L-ChT regimens [$OS_{1L\text{-}ChT}$ (95%CI): 10.7 (9.2–12.2) *vs.* 6.5 (3.8–9.2) months; $P = 0.043$; Δ 4.2 months; **Figure 4**]. Patients by the time of 1L-ChT

Predictor	Characteristic	*n* (%)	Events (%)	PFE - mOS1L-ChT (95% CI), months	other 1L-ChT - mOS1L-ChT (95% CI), months	*P*-value (log-rank)	Δ OS1L-ChT, months	*HR* (95% CI)	*P*-value
Sex	**Male**	67 (87.0)	65 (97.0)	9.4 (7.4 - 11.4)	5.5 (3.2 - 7.8)	0.132	3.9	0.73 (0.49 - 1.10)	0.135
Tobacco smoking	**≥ 30 packyears**	38 (49.4)	38 (100.0)	5.5 (1.6 - 9.4)	4.2 (2.0 - 6.4)	0.091	1.3	0.62 (0.35 - 1.09)	0.096
	Current smoker	67 (87.0)	66 (98.5)	9.1 (6.0 - 12.2)	5.1 (2.6 - 7.6)	0.100	4.0	0.72 (0.48 - 1.07)	0.103
Risk accumulation	**Tobacco and alcohol**	59 (76.6)	58 (98.3)	9.3 (6.0 - 12.6)	4.2 (2.7 - 5.7)	0.130	5.1	0.72 (0.47 - 1.11)	0.134
Alcohol consumption	**Current consumption**	55 (71.4)	54 (98.2)	9.4 (7.2 - 11.6)	5.5 (2.2 - 8.8)	0.178	3.9	0.74 (0.48 - 1.15)	0.182
Localization	**HNSCC outside Oropharynx**	45 (58.4)	44 (97.8)	10.7 (9.2 - 12.2)	6.5 (3.8 - 9.2)	**0.043**	4.2	0.63 (0.40 - 0.99)	**0.046**
	p16+ OPSCC and HNSCC outside Oropharynx	58 (75.3)	57 (98.3)	10.7 (9.3 - 12.2)	6.4 (3.9 - 8.9)	**0.039**	4.3	0.65 (0.43 - 0.98)	**0.041**
	OSCC	23 (29.9)	23 (100.0)	10.7 (9.3 - 12.1)	4.1 (2.4 - 5.8)	0.060	6.6	0.56 (0.30 - 1.04)	0.064
Extent of disease	**M1**	58 (75.3)	56 (96.6)	9.8 (7.7 - 11.9)	4.8 (3.3 - 6.3)	0.127	5.0	0.70 (0.44 - 1.11)	0.130
Enrollment in 1L-RCT	**Not enrolled**	43 (55.8)	41 (95.3)	9.3 (3.3 - 15.3)	4.1 (1.8 - 6.4)	**0.016**	5.2	0.45 (0.23 - 0.88)	**0.019**
Type of pretreatment	**Platinum-based ChT**	36 (46.8)	34 (94.4)	9.9 (8.3 - 11.5)	6.8 (0.0 - 13.8)	0.082	3.1	0.62 (0.36 - 1.07)	0.086
Number of pretreatments	**One pretreatment**	44 (57.1)	42 (95.5)	10.8 (6.6 - 15)	6.5 (1.3 - 11.7)	0.177	4.3	0.70 (0.41 - 1.18)	0.181
Age by the time of 1L-ChT	**≥ 60 years**	30 (61.0)	30 (100.0)	7.5 (1.6 - 13.4)	6.4 (3.6 - 9.2)	0.082	1.1	0.60 (0.34 - 1.07)	0.085

FIGURE 4 | Subgroups of incurable recurrent/metastatic head and neck squamous cell carcinoma benefitting from PFE administered according to the EXTREME protocol by prolonged $OS_{1L\text{-}ChT}$, demonstrated by Kaplan–Meier estimates applying log-rank tests and univariate Cox proportional hazard regression. *P* values of significant predictors <0.05 are in bold.

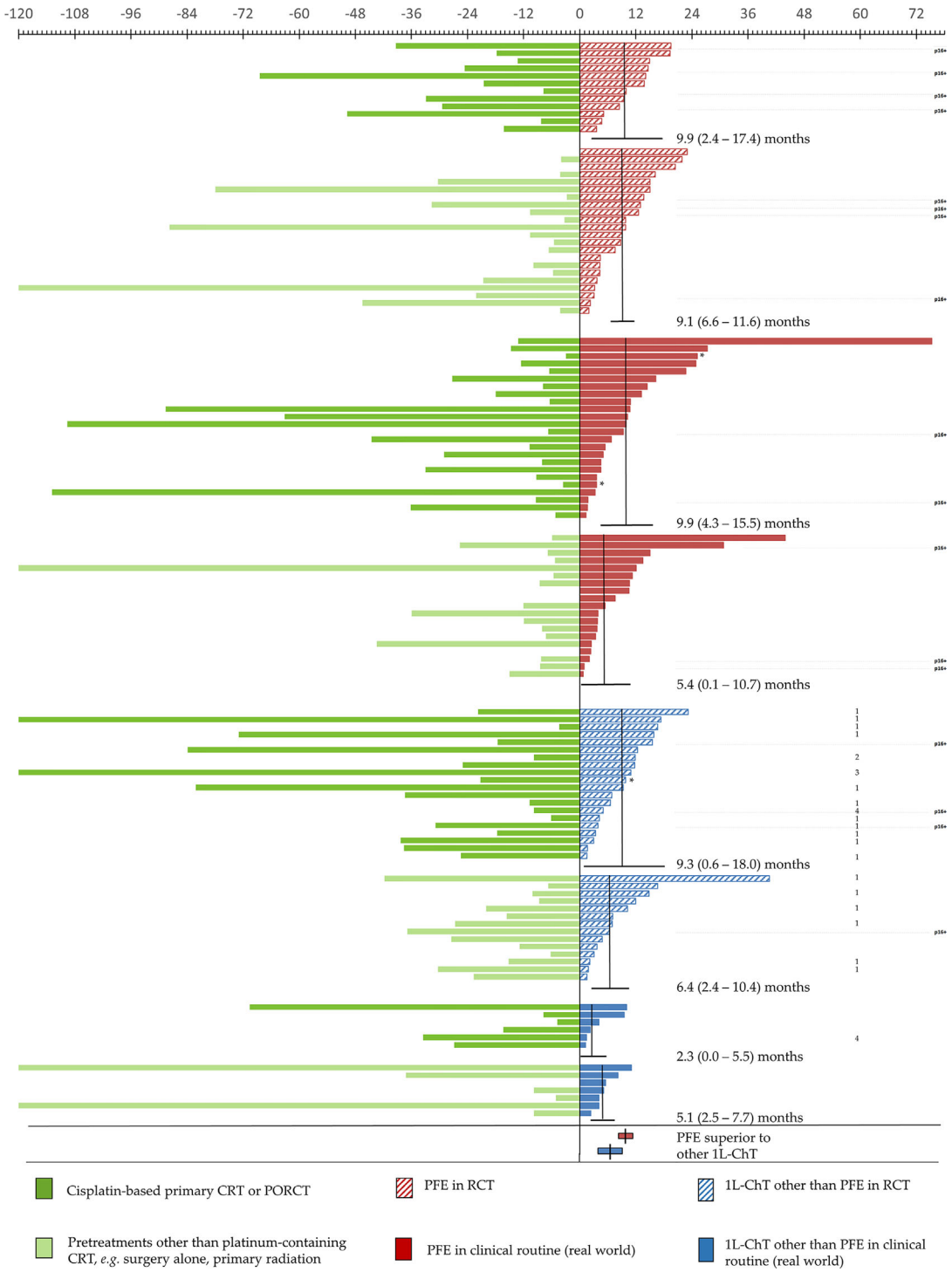

FIGURE 5 | Individual outcome of 124 patients with incurable recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) receiving various first-line chemotherapy regimens are depicted according to overall survival measured from diagnosis of R/M HNSCC til death ($OS_{1L\text{-}ChT}$). Patients are shown sorted stratified according to 1L-ChT, either EXTREME-regimen (PFE, red; *n* = 77) or other 1L-ChT (blue; *n* = 47) and treatment either within randomized controlled trial (RCT; shaded) or in clinical routine ("real world setting", full). Type of prior treatment in curative attempt is indicated in dark green (cisplatin-based chemo-radiation (CRT) or post-operative radio-chemotherapy (PORCT)) *vs*. light green (other or no pretreatment); time from initial diagnosis of HNSCC until diagnosis of incurable disease requiring 1L-ChT is shown in the left panel, $OS_{1\text{-}ChT}$ in the right panel according to the upper scale showing time in months. The horizontal lines indicate $mOS_{1\text{-}ChT}$ (95% confidence interval). Median and 95%CI of $OS_{1l\text{-}ChT}$ of PFE *vs*. other 1L-ChT in the total cohorts are shown in the lower rows. *censored: alive at last follow-up (*n* = 3); 1, CeFCiD (6); 2, ADVANTAGE (7); 3 RESGEX (8); 4, TPExtreme (9); p16+, p16+ OPSCC.

diagnosed with distant metastasis (M1) demonstrated an improved benefit from PFE compared to patients with loco-regional recurrence (**Figure 4**). However, we performed sensitivity analyses and excluded all patients that were diagnosed already in a locally very advanced and metastatic stage without any curative option and therefore receiving 1L-ChT as first treatment (n = 10). Kaplan–Meier estimates showed a $mOS_{1L\text{-}ChT}$ after PFE *vs.* other 1L-ChT of 9.4 (95%CI: 7.8–10.9) months *vs.* 6.5 (95%CI: 4.1–9.2) months for the remaining 114 patients (P = 0.163). This compares well to the $OS_{1L\text{-}ChT}$ for the total cohort.

The lifestyle-factors tobacco and alcohol showed an impact on outcome (**Figure 4**). There were patients with both risk factors (current or former alcohol consumption and tobacco smoking; n = 93) and those without or solely one risk factor (n = 26; five patients without information). Both groups demonstrated a benefit from PFE, patients with two risk factors had an impaired $mOS_{1L\text{-}ChT}$ but showed a higher benefit from PFE in Kaplan–Meier estimates [$mOS_{1L\text{-}ChT}$ (95% CI): 9.3 (6.0–12.6) *vs.* 4.2 (2.7–5.7) months; P = 0.130; **Figure 4**]. We found a significant correlation of double-positive risk factor-anamnesis with two baseline characteristics: young patients (≤60 years at 1L-ChT; *Pearson's* r = 0.272; P = 0.003) and male patients (*Pearson's* r = 0.288, P = 0.002) did more often belong to the group with both risk factors.

Multivariate Cox Proportional Hazard Regression for Outcome

The MCR model for OS in the total cohort achieving highest significance (X^2 = 21.7, P = 0.001) included five independent risk factors: the number of pretreatments and pack years smoking history, alcohol consumption status, index HNSCC of the oropharynx, and type of 1L-ChT (**Figure 6**). Bootstrapping revealed these factors to be predictive for $OS_{1L\text{-}ChT}$. The stepwise forward method for building the MCR failed to detect any predictive value of the patient's age by the time of 1L-ChT, TNM at first diagnosis or even enrollment in a first-line RCT for $OS_{1L\text{-}ChT}$. Interestingly, having a p16+ OPSCC was also not predictive for improved outcome, and MCR including either p16 positivity or p16 negativity as covariate had reduced significance compared to MCR including OPSCC as covariate; therefore OPSCC summarizing p16+ and p16- OPSCC remained in the MCR.

In MCR model for the PFE subgroup (n = 77; X^2 = 15.0, P = 0.002), three risk factors were found to be predictive for $OS_{1L\text{-}ChT}$ after PFE. Cisplatin-based CRT/PORCT prior to PFE (*HR* (95% CI): 0.67 (0.42–1.09); P = 0.106) was beneficial, index OPSCC (*HR* (95%CI): 1.61 (0.97–2.68); P =0.066) and alcohol consumption ≥ 30 g/d (*HR* (95%CI): 2.04 (1.22–3.41); P = 0.007) predicted impaired $OS_{1L\text{-}ChT}$.

Identification of PFE Long-Term Survivors

Figure 5 shows individual $OS_{1L\text{-}ChT}$ in R/M HNSCC stratified according to PFE *vs.* other 1L-ChT either in treatment within RCT or in clinical routine providing "real world evidence". According to identification of prior cisplatin-based CRT or PORCT as significant $OS_{1L\text{-}ChT}$ predictor, we further stratified these groups by cisplatin-based CRT or PORCT *vs.* other pretreatments. The improved outcome of certain PFE-treated R/M HNSCC patients allowed further investigations in the subgroup surviving more than 11.3 months, the upper bound of 95%CI for $mOS_{1L\text{-}ChT}$ in PFE-treated patients. These 28 individuals had a median age (57.3 years) comparable to the total cohort of PFE patients (n = 77, 56.6 years). They were quite similar to the total PFE cohort respective to sex (17.9% female), type of prior treatment (50% cisplatin-based CRT/PORCT) besides slightly lower median exposure to risk factors (22 pack years in 64.3% current smokers, as well as 64.3% current alcohol consumers; **Table 1**). Even the 12 RWE-PFE patients with $OS_{1L\text{-}ChT}$ above 95%CI $OS_{1L\text{-}ChT}$ in the CRT/PORCT and "other" subgroups (10.7 and 15.5 months, respectively) had a similar median age at the time of initial diagnosis of HNSCC (57.4 years) compared to median age in the PFE subgroup (56.6 years, **Table 1**). Eleven of these 12 patients received PFE at first recurrence, one (8.3%) was treated with PFE at second recurrence. As one curative treatment prior to any 1L-ChT is

Predictor	Characteristic	n (%)	events (%)	HR (95% CI)	P-value	P-value bootstrapping
Number of pretreatments	**One pretreatment [A]**	66 (53.2)	64 (96.7)	0.582 (0.391 - 0.865)	**0.007**	**0.007**
Type of 1L-ChT	**PFE [B]**	77 (62.1)	75 (97.4)	0.720 (0.476 - 1.064)	0.107	0.087
Alcohol consumption	**≥ 60g/d [C]**	37 (29.8)	37 (100.0)	1.470 (0.937 - 2.313)	0.095	0.058
Tobacco smoking	**≥ 30 packyears [D]**	58 (46.8)	58 (100.0)	1.621 (1.041 - 2.451)	**0.027**	**0.027**
Localization and p16-status	**p16-negative OPSCC [E]**	25 (20.2)	24 (96.0)	1.425 (0.868 - 2.34)	0.161	0.153

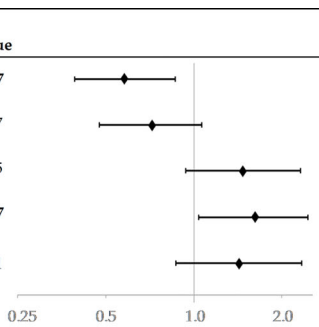

FIGURE 6 | Predictors in multivariate Cox proportional hazard regression (HR) and 2-sided P-values from internal validation using bootstrapping applying 1,000 iterations. Significant independent predictors P <0.005 are in bold. [A] Reference: 1L-ChT at initial diagnose or ≥2 pretreatments; [B] Reference: other 1L-ChT regimen; [C] Reference: <60 g/d; [D] Reference: <30 pack years; *E* Reference: HNSCC outside oropharynx.

an independent predictor for improved $OS_{1L\text{-}ChT}$ in the total cohort, this might be causative involved in their prolonged $OS_{1L\text{-}ChT}$. However, only 2/12 (16.7%) of RWE-PFE long-term survivors had a current alcohol consumption >30 g/d, pointing to the absent detrimental impact of maintained alcohol consumption on $OS_{1L\text{-}ChT}$ in most of RWE-PFE long-term survivors. Interestingly, smoking history and adhering to tobacco smoking may also play a role as only seven of these 12 long-term survivors (58.3%) were current smokers, and the median cumulative nicotine exposure was 25 pack years and somewhat lower compared to the total PFE cohort (**Table 1**). The proportion of p16+ OPSCC was higher in RCT; their $OS_{1L\text{-}ChT}$, however, was not superior compared with R/M HNSCC localized outside the oropharynx (**Figure 3C**).

Identification of Long-Term Survivors in Other 1L-ChT Regimens

As enrollment in RCT was predictive for improved $OS_{1L\text{-}ChT}$ only in 34 *vs.* 13 patients (see *$OS_{1L\text{-}ChT}$ after other 1L-ChT regimen*) we were interested in long-term survivors in this subgroup. According to numbers in the right panel of **Figure 5**, PFE-based regimens containing an additional (investigational) drug, for instance docetaxel (TPFE) in the CeFCiD trial [labeled 1 (6)], cilengitide in the ADVANTAGE trial [labeled 2 (7)], or replaced cetuximab by glycosylation-modified cetuximab in the RESGEX trial [labeled 3 (8)], long-term survivors were only seen after PFE-based 1L-ChT. However, the outcome observed in such intensified PFE-based 1L-ChT did not improve outcome in general at least in our cohort as it is obvious that a huge heterogeneity exists in this regard.

DISCUSSION

According to several lines of evidence, our monocentric study comprises a sufficient number of R/M HNSCC receiving 1L-ChT to show outcome differences dependent on a number of well-defined covariates. The $mOS_{1L\text{-}ChT}$ in our sample is comparable to the survival times found in prior trials (6, 7, 15). Therefore, the subgroups with and without benefit from PFE identified in our study confirm the existence of certain subgroups already described (3). Uni- and multivariate analyses demonstrated that the number of pretreatments, consumption of alcohol and/or tobacco smoking as well as localization of the index cancer and patients' age have a certain effect on $OS_{1L\text{-}ChT}$. When treated with PFE in particular, predictive covariates are mostly the same. However, our study provides evidence that prior intensified treatments making use of cisplatin-based CRT and especially cisplatin-based PORCT do not negatively affect survival in PFE but rather improve $OS_{1L\text{-}ChT}$. Indeed, prior cisplatin-based CRT or PORCT appeared to be an additional independent predictor for significant prolonged $OS_{1L\text{-}ChT}$. These findings from multivariate Cox regression analyses may contribute to the ongoing discussion about a potential negative impact of treatment escalation in the curative setting on further therapies and the possibility to re-challenge R/M HNSCC with cisplatin when progressing after cisplatin-based curative treatment. As the median time from curative treatment with surgery followed by cisplatin-based PORCT to 1L-ChT (n = 52) was 30.6 months (95%CI: 21.5–40.2) and substantially longer (P = 0.005) compared to 10.6 months (95%CI: 5.0–16.3) of patients without prior treatment or other types of prior curative treatment, and these cisplatin-pretreated R/M HNSCC patients had the highest benefit from PFE, treatment escalation in presence of risk factors in the curative setting improves outcome and does not reduce $OS_{1L\text{-}ChT}$ if PFE is used. As, additionally, a 2L-ChT could be applied in a higher frequency after PFE as compared to other 1L-ChT, treatment escalation in the curative setting *via* cisplatin-based PORCT whenever high risk for relapse/recurrent disease (more than two disease-positive neck nodes, extracapsular extension of neck nodes, positive or narrow resection margins below 5 mm) is detected appears to be warranted.

Our results confirm OS data for PFE including subgroup analyses obtained in the landmark phase-III RCT EXTREME (3). Comparing outcome of PFE with PF, Vermorken et al. (3) showed in univariate models that patients ≥ 65 years demonstrate a minor benefit from PFE compared to younger patients. Our retrospective study comprises only 21 *vs.* 15 R/M HNSCC patients ≥65 years receiving PFE *vs.* other 1L-ChT regimen. We have not seen an inferiority of PFE in this subgroup compared to patients <65 years. By performing this analysis with a slightly different cut-off point of 60 years (30 *vs.* 15 patients), we found no evidence for an inferiority of PFE neither.

The recently published ELAN-FIT trial by Guigay et al. (16) showed a $mOS_{1L\text{-}ChT}$ of 14.7 months (95%CI: 11.0–18.2) after PFE for patients aged 70 and older and ECOG performance status 0 or 1. The impact of age and its influence on PFE efficacy and risk will be probably important in future trials. However, we found no evidence in our cohort for calendar age alone being the most relevant eligibility criterion for PFE, provided good general health (ECOG 0 or 1). PFE is only approved for ECOG 0 and 1 patient presenting, so the MDTB made the decision for either offering participation in a 1L-ChT RCT or 1L-ChT treatment in the routine setting only provided good general health as reflected by ECOG 0 or 1. Consequently, our sample mainly included "fit" patients in our retrospective trial to ensure comparability. As Guigay et al. (17) showed, "unfit" patients may be eligible for PFE or comparable regimens after a comprehensive geriatric assessment. By performing RCTs after a geriatric assessment, there could be more evidence about the impact of calendar age *vs.* biological age on treatment eligibility and potential benefit in older patients.

Referring to Guigay et al. (9), the TPExtreme (TPE; docetaxel, cis- or carboplatin, cetuximab) 1L-ChT regimen is beneficial when followed by ICB in 2L-ChT. As retrospectively found, TPE outperformed PFE only in this treatment sequence. Due to our small sample of 124 patients collected over years and only two TPE patients unfit to receive 2L-ChT after recurrence, there are no such patients in our cohort.

Patients in the PFE subgroup had a longer $mOS_{1L\text{-}ChT}$ – independently on the following 2L-ChT— than patients treated with other 1L-ChT in our analysis. As all studies demonstrated the lasting value of PFE, we recommend—against the often suggested alternative use of TPE as unproblematic replacement for PFE to avoid potential dihydropyrimidine dehydrogenase-(DPD-) toxicity—rather DPD testing according to established guidelines (18) so that R/M HNSCC patients still can benefit from PFE. As only one RCT demonstrated an improved OS of R/M HNSCC in the minor subgroup of patients treated sequentially first with TPE followed by ICB over PFE followed by ICB in a retrospective analysis (17), it might be too soon to change 1L-ChT of R/M HNSCC in absence of a positive phase III RCT demonstrating superiority of TPE over PFE. Moreover, we were unable to see a benefit from TPE as only 2/124 patients received TPE, and both (indicated with four in **Figure 5**) had a rather impaired outcome below the $mOS_{1L\text{-}ChT}$. Without replication of the findings by Guigay et al. (9) in such a phase-III RCT the TPExtreme-ICB treatment sequence so far remains experimental at best.

Today, no published data for the efficacy of PFE for R/M HNSCC progressing under 1L-ICB are available. The question if patients failing on curative treatment involving ICB thereafter progressing and requiring 1L-ChT should preferentially be treated with PFE is not yet completely clear. However, we expect that PFE can benefit a substantial proportion of such R/M HNSCC.

Regarding the influence of HPV-status on $OS_{1L\text{-}ChT}$, we have seen an impaired $OS_{1L\text{-}ChT}$ in patients suffering from a p16-negative OPSCC compared to patients with a p16+ OPSCC or index HNSCC outside the oropharynx. This is in line with former findings (19, 20). Based on the study by Mehra et al. (19) showing an improved OS in p16+/HPV+ R/M HNSCC patients, Vermorken et al. (20) performed a retrospective analysis of data from the EXTREME trial (3) and found a p16+/HPV-prevalence and p16+/HPV-related $OS_{1L\text{-}ChT}$ similar to our findings. There is an ongoing discussion about the influence of HPV on survival in R/M HNSCC. In contrast to Mehra et al. and Vermorken et al., Szturz et al. (21) found in a meta-analysis of four prospective RCT that HPV-related (p16+ or HPV-DNA+) tumors barely responded to EGFR-directed monotherapy, whereas improved response rates were only observed in HPV-negative cases. Since we did not observe detrimental effects by p16 positivity on $OS_{1L\text{-}ChT}$ no matter if EXTREME or other regimens were applied, but $OS_{1L\text{-}ChT}$ was strongly reduced in oropharyngeal R/M HNSCC and even further reduced in p16-negative cases, our study highlights the importance of further investigations in this field. The poorest $OS_{1L\text{-}ChT}$ in oropharyngeal R/M HNNSCC could be linked to the proximity to essential cervical structures including arteries and their infiltration. Therefore, R/M HNSCC with rather reduced infiltrating growth patterns and without vascular infiltration may have prolonged $OS_{1L\text{-}ChT}$ independent from being HPV-related. Additionally, distance of the R/M HNSCC from vital vessels might prolong the time to life-threatening destruction of indispensable organs and critical bleeding events including arterial blowout leading to death.

During the time period analyzed in this retrospective study, therapy guidelines for R/M HNSCC have changed. Nowadays, and according to KEYNOTE-048 trial (4), immune checkpoint blockade (ICB) by pembrolizumab is declared new standard of care for patients with CPS >20 or ICB-PF combination for patients with CPS >1 to ≤20. According to KEYNOTE-048 investigators, PFE remains standard of care for CPS ≤1. Consequently, PFE may be 1L-ChT standard for this subgroup and 2L-ChT option for patients progressing after ICB. However, as we confirm data from the EXTREME trial (3), especially male patients, subgroups accumulating more lifestyle-associated risk factors, and those with their index HNSCC outside oropharynx still benefit the most from PFE. KEYNOTE-048 subgroup analyses (4) addressed this issue showing that patients <65 years and ≥65 years do not differ in benefit from pembrolizumab ± chemotherapy. There was no significant difference between never and former/current smokers. It may be interesting to conduct further analyses to see if there are any differences in OS depending on patients' characteristics described here (**Figure 5**).

Unlike ICB in the KEYNOTE-048 trial, ICB with durvalumab (PD-L1 inhibitor) ± tremelimumab (CTLA-4-inhibitor) in the KESTREL phase III trial failed to meet the primary endpoint of improved OS compared to PFE. As AstraZeneca reported this result just recently [2021-02-05 (22)] and a peer-reviewed paper on KESTREL is still not published, it might be too soon to rank any ICB in general over PFE. At least, PFE should be considered standard for all 1L-ChT not belonging to the CPS >1 subgroup of R/M HNSCC patients.

Argiris et al. (23) showed an improved response rate and progression-free survival by adding the anti-VEGF antibody bevacizumab to chemotherapy. This may provide evidence for a benefit by targeted therapies other than EGFR- or PD-L1-inhibitors combined with PF. However, acute toxicity appeared to be increased if PF and bevacizumab were used in 1L-ChT, and the gain in OS compared to PF rather limited (18).

Discussing their KEYNOTE-048 results and referring to retrospective trials (24, 25), post-pembrolizumab sensitization of R/M HNSCC to a subsequent therapy with PFE was mentioned by Burtness et al. (4). This highlights the potential importance of 2L-PFE applied after 1L-ICB in the future. In the light of ICB applied within multimodal treatment regimen in the curative setting, *e.g.* during induction-chemotherapy for larynx-organ preservation or ICB as component of adjuvant therapies after curative resection and in postoperative maintenance, we are convinced that PFE will have a dominant role as 1L-ChT also in the future (26, 27). In context of earlier investigations highlighting improved outcome after increased utilization of PORCT in treatment of L/HSCC (23), prolonged $OS_{1L\text{-}ChT}$ through PFE after cisplatin-based PORCT may at least partially have contributed to the welcome impact of indication shift towards increased use of cisplatin-based PORCT according to Bernier and Cooper (12, 13) on heightened OS time (28).

In our study, 14.3% of the patients died within 3 months after starting PFE. These figures compare well to 17.1% found by Vermorken et al. (3). The majority of early deaths observed in

our cohort occurred outside RCTs (72.7% *vs.* 27.3% of all fatalities during PFE treatment). Treatment in clinical routine apart from adherence to the complete checklist of eligibility criteria as required to enter any of the RCT as well as survivorship bias may have potentially contributed to this situation. However, outcome in RCT *vs.* "real world" was not significantly different overall. Reproducibility of survival benefit of certain subgroups independent from RCT participation shows that RCT results are representative for the outcome achieved by PFE even in clinical routine. Subgroup analyses of the seven long-term survivors within the subgroup of RWE-PFE treated patients allude to the impact of risk factors on survival. Those seven patients barely drank alcohol but received PFE after cisplatin-based CRT/PORCT. The overall well-comparable or even slightly improved outcome in RWE compared to RCT PFE-treated R/M HNSCC patients demonstrates an unprecedented translation of findings from RCT into routine results with high concordance.

Only a minority of R/M HNSCC patients treated in other 1L-ChT RCT demonstrated superior $OS_{1l\text{-}ChT}$ from a further intensified PFE-based regimen, whereas most had inferior $OS_{1l\text{-}ChT}$ compared to PFE (**Figure 5**). However, the only long-term survivors detected among other 1L-ChT received an intensified PFE-based regimen. Unfortunately, the frequency of patients without benefit from treatment escalation was found to be higher than those with prolonged $OS_{1L\text{-}ChT}$. Increased toxicity as reported also in (6) and (7) may have essentially contributed to this finding by causing detrimental effects. Further investigations to distinguish long-term survivors and those unsuitable for treatment escalation beyond the use of PFE appear to be warranted.

There are limitations of our study. Our retrospective monocentric study involved 124 R/M HNSCC patients including 77 treated with PFE. However, this case number was sufficiently large enough to elucidate some independent predictors for outcome and to confirm the existence of the earlier described subgroups of R/M HNSCC patients. Moreover, we did not find any significant survival differences between patients receiving PFE in- or outside the numerous first-line RCT arguing for a representative mixture of patients that at least in our clinic remained stable over two decades, a consistency in decision-making for usage of PFE in 1L-ChT for R/M HNSCC, and improved outcome achieved through PFE. Therefore, the effects detected in our sample demonstrate stability over time and confirm the initial findings from the EXTREME trial (3) being representative for good outcome after PFE in general. A strength of our study is the complete follow-up and the multivariate analyses including bootstrapping for internal validation of independent predictors to avoid over-optimism in interpretation of our findings.

CONCLUSIONS

This retrospective study highlights the lasting value of the triplet cisplatin, 5-fluoruracil, cetuximab (PFE) not only as comparator treatment within randomized controlled trials (RCT) but also—and independent on the age of R/M HNSCC patients—in clinical routine. Interestingly, we found no evidence for a negative impact of prior intensified treatments making use of primary or postoperative cisplatin-based chemo-radiotherapy on overall survival following first-line chemotherapy but rather improved outcome in this subgroup achieved by PFE independent from participation in RCT or applied in the "real world" setting. Demonstrating again the high value of PFE in first-line chemotherapy, this effective treatment should not be replaced by treatments that failed to demonstrate superiority in RCT. PFE should hence remain standard for first-line chemotherapy at least in patients not belonging to the well-defined subgroups of recurrent/metastatic head and neck squamous cell carcinoma eligible for pembrolizumab or PF plus pembrolizumab according to KEYNOTE-048 (4).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Human Ethics Committee of the University Leipzig (votes 201-10-12072010 and 202-10-12072010). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, GW. Data curation, KL, MP, TW, and GW. Formal analysis, KL and GW. Investigation, KL and GW. Methodology, GW. Project administration, GW. Resources, AD and GW. Validation, GW. Visualization, KL and GW. Writing – original draft, KL and GW. Supervision, SW, AD, VZ, and GW. Writing – review and editing, KL, MP, TW, SW, AD, VZ, and GW. All authors contributed to the article and approved the submitted version.

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The Role of Akt in Acquired Cetuximab Resistant Head and Neck Squamous Cell Carcinoma: An *In Vitro* Study on a Novel Combination Strategy

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The epidermal growth factor receptor (EGFR) is a therapeutic target in head and neck squamous cell carcinoma (HNSCC). Resistance to EGFR-targeted therapies, such as cetuximab, poses a challenging problem. This study aims to characterize acquired cetuximab resistance mechanisms in HNSCC cell lines by protein phosphorylation profiling. Through this, promising combination treatments can be identified to possibly overcome acquired cetuximab resistance in HNSCC. Protein phosphorylation profiling showed increased phosphorylation of Akt1/2/3 after cetuximab treatment in acquired cetuximab resistant cells compared to cetuximab sensitive cells, which was confirmed by western blotting. Based on this protein phosphorylation profile, a novel combination treatment with cetuximab and the Akt1/2/3 inhibitor MK2206 was designed. Synergy between cetuximab and MK2206 was observed in two cetuximab sensitive HNSCC cell lines and one acquired cetuximab resistant variant in simultaneous treatment schedules. In conclusion, this study demonstrates that increased Akt1/2/3 phosphorylation seems to be characteristic for acquired cetuximab resistance in HNSCC cell lines. Our results also show an additive to synergistic interaction between cetuximab and MK2206 in simultaneous treatment schedules. These data support the hypothesis that the combination of cetuximab with PI3K/Akt pathway inhibition might be a promising novel therapeutic strategy to overcome acquired cetuximab resistance in HNSCC patients.

Keywords: PI3K/Akt pathway, cetuximab, EGFR, HNSCC, Akt inhibitor, resistance

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer type worldwide and remains one of the most challenging malignancies to treat (1, 2). Through our increasing knowledge regarding the molecular biology of HNSCC, several therapeutic strategies have been developed. The introduction of targeted therapies that inhibit oncogenic signaling pathways, as well as the development of immunotherapies that activate a patient's immune system are now at the forefront of personalized medicine in cancer treatment.

Over the past decades, research has revealed that the epidermal growth factor receptor (EGFR, HER1) plays an integral role in the tumorigenesis of HNSCC. Increased or sustained activation of the EGFR signaling pathway can induce malignant transformation through sustained signaling for cell proliferation, anti-apoptotic signaling, angiogenesis and metastasis (3). Furthermore, EGFR is highly expressed in a wide range of malignancies, including HNSCC. As a result, EGFR is considered as a compelling drug target (3, 4).

In 2006, the anti-EGFR monoclonal antibody (mAb) cetuximab, was approved by the Food and Drug Administration (FDA) in combination with radiotherapy for locoregionally advanced HNSCC (median overall survival (OS) of 4.1 years versus 2.4 years with radiotherapy alone) and in combination with platinum-based therapy and infusional 5-fluorouracil (EXTREME regimen) for the first-line treatment of patients with recurrent/metastatic (R/M) HNSCC (median OS 10.1 months versus 7.4 months with chemotherapy alone) (5–7). To date, the therapeutic landscape of HNSCC is changing as the anti-programmed death-1 (PD-1) immune checkpoint inhibitor pembrolizumab has been FDA-approved in June 2019 as a first-line treatment of R/M HNSCC. The Keynote-048 study demonstrated that pembrolizumab with platinum and 5-fluorouracil significantly improved median OS versus the EXTREME regimen with cetuximab in the total population of HNSCC patients (13.0 months versus 10.7 months) (8). In particular, patients expressing programmed death ligand-1 (PD-L1) (85% of HNSCC patients has combined positive score ≥ 1) show an increased benefit from treatment with pembrolizumab plus platinum and 5-fluorouracil (median OS 13.6 months versus 10.4 months). However, response rates to this treatment regimen are 36% and thus still limited and comparable to the EXTREME regimen with cetuximab (8, 9). Hence, to date, management of R/M HNSCC relies on combination treatment involving platinum, 5-fluorouracil and the addition of pembrolizumab or cetuximab. However, as mentioned, only a fraction of HNSCC patients respond to these treatment regimens.

The reason for resistance to pembrolizumab treatment in HNSCC remains largely unknown. In this regard, several biomarkers, such as PD-L1 expression, immune infiltration, tumor mutational burden and immune-gene expression profiling, have been explored for their predictive potential. However, none of them could be validated in HNSCC so far (10). Concerning cetuximab, this resistance is partly attributable to lack and/or loss of sensitivity of tumor cells to EGFR inhibition, which develops during treatment and compromises the therapeutic outcome. If resistance to cetuximab therapy is present at baseline, this is defined as intrinsic (primary) resistance and can be explained by resistance-conferring factors pre-existing in the bulk of tumor cells. Moreover, nearly all patients whose tumors initially respond inevitably become acquired (secondary) resistant. Acquired resistance refers to disease progression during ongoing treatment that was initially effective (11). In these scenarios, targeting EGFR alone may not be efficacious and requires the addition of a supplementary targeting agent to maximize the therapeutic response.

Therefore, the present study aims to investigate a novel combination strategy to overcome acquired cetuximab resistance. We hypothesized that acquired cetuximab resistance in HNSCC may arise from the activation of compensatory signaling pathways following cetuximab treatment, which are able to reverse the inhibitory effects of cetuximab through phosphorylation of key proteins, thereby promoting cell survival. Pharmacological inhibition of these phosphorylated proteins might be essential to overcome acquired cetuximab resistance in HNSCC. Therefore, we first characterized the protein phosphorylation profile of two cetuximab sensitive (Cet_{Sens}) HNSCC cell lines and their acquired cetuximab resistant (Acq_{Res}) variants. Based on this protein phosphorylation profile, a novel combination treatment was designed to overcome acquired therapy resistance.

MATERIAL AND METHODS

Cell Culture

HPV-negative human HNSCC cell lines SC263 and SCC22b were kindly provided by Prof. Dr. Sandra Nuyts (University Hospital Leuven, Leuven, Belgium) and Prof. Dr. Olivier De Wever (Laboratory of Experimental Cancer Research, Ghent University Hospital, Ghent, Belgium). These HNSCC cell lines were previously identified as Cet_{Sens} following cetuximab treatment for 168h using the colorimetric sulforhodamine B (SRB) assay. Isogenic Acq_{Res} HNSCC cell lines (SC263-R and SCC22b-R) were previously generated by chronically exposing these initially Cet_{Sens} cell lines (parental) to cetuximab, starting with the IC_{50} concentration of cetuximab (12–14). In parallel, control cell lines (SC263-S and SCC22b-S) were established by exposure to the vehicle control (PBS). After 10 dose doublings, dose-response studies demonstrated that cetuximab-exposed cells developed resistance towards cetuximab (12–14). All cell lines were cultured in DMEM, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine (Life Technologies, Merelbeke, Belgium). Resistant cell lines were exposed to the highest doubling dose of cetuximab every four weeks. Cells were grown as monolayers and maintained in exponential growth in 5% CO_2/95% air in a humidified incubator at 37°C. All cell lines were confirmed free of mycoplasma infection through regular testing (MycoAlert Mycoplasma Detection Kit, Lonza, Verviers, Belgium). The identity of each cell line was validated through short tandem repeat profiling (Centre of Medical Genetics, University of Antwerp, Antwerp, Belgium).

Human Protein Phosphorylation Antibody Array

Human phospho-kinase antibody array kit (ARY003B, Proteome Profiler, R&D Systems, Minneapolis, MN, USA) was used to determine the relative levels of protein phosphorylation in the Cet_{Sens} and Acq_{Res} cell lines after cetuximab treatment. The human phospho-kinase antibody array was performed according to the manufacturer's protocol. In short, cells were lysed on 6-well plates after treatment with cetuximab (0 and 100 nM, 2 hours, diluted in sterile PBS, Merck, Darmstadt, Germany). Twenty minutes before lysis, epidermal growth factor (EGF, 50 ng/ml, Sigma-Aldrich, Diegem, Belgium) was added to the medium. Protein concentrations were determined using the Pierce BCA protein kit (Thermo Scientific, Erembodegem, Belgium). Next, samples were prepared and 450 µg of proteins were incubated overnight with nitrocellulose membranes which are spotted with 46 capture antibodies in duplicate. The specific target proteins (**Supplementary Table 1**), if present in the sample, bind to these capture antibodies, leading to protein-antibody interactions. These protein-antibody interactions are then visualized using chemiluminescent detection reagents on the Lumi-Imager (Roche Diagnostics, Vilvoorde, Belgium). The antibodies of this kit bind to all isoforms of the target proteins and are therefore not isoform-specific. The signal is proportional to the amount phosphorylation in the bound analyte. Quantification of phosphorylation levels was executed following the manufacturer's protocol. In short, the integrated optical density of each spot was measured and corrected for background signal using Image J software (15). Only proteins that gave rise to an integrated optical density at least 1.5-fold above background were further used for data processing. Mean integrated optical densities were obtained by averaging the integrated optical density of the duplicates on the array. The fold changes were calculated by dividing the mean integrated optical densities of the treatment and control groups.

Western Blot

In order to validate findings of the human phospho-kinase antibody array with western blot, cell lysates were three times independently prepared as described above. Twenty micrograms of proteins were separated by SDS-page (10% polyacrylamide gel, 100V, 2h) and western blot was performed (100V, 1h). Blocking of non-specific binding sites was done by incubation of the membrane with Odyssey blocking buffer TBS (Li-Cor, Leusden, The Netherlands) for 1h at room temperature while shaking. Next, primary and secondary antibody incubation and washing was performed using the SNAP id 2.0 protein detection system (Merck Millipore) according to the manufacturer's instructions. Membranes were incubated with the following antibodies: phospho-Akt (S473) rabbit mAb (1:1000, no. 193H12, Cell Signaling Technology, Leiden, The Netherlands) and total Akt (pan) rabbit mAb (1:1000, no. 11E7, Cell Signaling Technology). Anti-β-actin was used as an internal standard (1:5000, no. A5441, Sigma Aldrich). Goat-anti-rabbit (1:10000, no. 926-32211, Li-Cor) or goat-anti-mouse (1:10000, no. 926-68070, Li-Cor) fluorescently labeled secondary antibodies were used and fluorescent detection was performed using the Odyssey imaging system (Li-Cor). Protein levels were quantified using Image Studio™ Lite, a software program available on the Odyssey imaging system. Phospho-Akt and Akt expression levels were corrected for loading differences based on beta-actin expression. Fold changes were calculated by dividing the mean fluorescent signal of the treatment and control groups.

In Silico Analysis of Akt1, Akt2 and Akt3 Expression

The baseline mRNA expression of Akt1/2/3 in HNSCC patients was examined using the RNA sequencing data from The Cancer Genome Atlas (TCGA) dataset (Provisional, 522 sequenced HNSCC patients). RNASeqV2 from TCGA was processed and normalized using the software package RNA-Seq by Expectation Maximization (RSEM) in order to generate transcripts per million. This dataset was downloaded from cBioportal.

Sulforhodamine B Assay

Cell survival was assessed using the colorimetric SRB assay, as previously described (16, 17). This assay is used for cell density determination, based on the measurement of cellular protein content, whereby it is not possible to make a distinction between inhibition of proliferation (cytostatic effect) and cell death (cytotoxic effect). Optimal seeding densities for each cell line were determined in order to ensure exponential growth during the whole duration of the assay. Cells were counted with a TC20 Automated Cell Counter (Biorad, Temse, Belgium). After overnight incubation, cells were treated with MK2206 (72h, 0-5 µM, pan-Akt inhibitor, Selleck Chemicals, Houston, USA) in combination with cetuximab (0-50 nM). Hereby, two simultaneous combination schedules were tested:

1. Cetuximab plus MK2206 with total treatment duration of 72h
2. Cetuximab for 168h with MK2206 added during the last 72h of treatment

MK2206 was diluted in DMSO (Merck Millipore SA/NV, Overijse, Belgium) and further dilutions were made in cell culture medium. IC_{50} values (i.e. drug concentration causing 50% growth inhibition) were calculated using Graphpad Prism 9 software. Possible synergism between cetuximab and MK2206 was determined by calculation of the combination index (CI) using the Additive Model as described by others (18–20). CI < 0.800, CI = 1.000 ± 0.200 and CI > 1.200 indicated synergism, additivity and antagonism, respectively.

Statistical Analysis

We performed all experiments at least three times independently, unless otherwise stated. In cytotoxicity experiments, each condition was tested in triplicate in each of the three experiments. Differences in total and phosphorylated Akt1/2/3, determined with western blot, were statistically analyzed using the non-parametric Kruskal-Wallis test. Significant values were adjusted by the Bonferroni correction for multiple testing. One-way ANOVA with Tukey-Kramer HSD posthoc analysis was

used to assess significant differences in Akt1/2/3 mRNA expression of HNSCC patients (RNASeqV2 TCGA data). The influence of cetuximab treatment on the effect of MK2206 alone was evaluated for each cell line using linear mixed models in case of non-independent observations. More specifically, combination treatment was set as fixed effect. A random intercept for experimental replicates was added in order to account for the dependence of observations between experiments. The influence of cetuximab resistance status on the effect of MK2206 alone was also evaluated using linear mixed models. Resistance status was set as fixed effect and a random intercept for cell line and experimental replicates was added to account for the dependence of observations between cell lines and experiments. GraphPad Prism 9 was used for data comparison and artwork. All statistical analyses were performed in JMP Pro 15 and SPSS v25. P-values below 0.050 were considered significant.

RESULTS

Acquired Cetuximab Resistant HNSCC Cell Lines Show Increased Phosphorylation of Akt After Cetuximab Treatment

In order to characterize acquired cetuximab resistance, a protein phosphorylation profile was established using the human phospho-kinase antibody array. Hereby, the effect of cetuximab treatment on the phosphorylation of various proteins (**Supplementary Table 1**) was determined in two Cet_{Sens} HNSCC cell lines and their isogenic Acq_{Res} variants. Data analysis of the protein phosphorylation profile revealed a differential response of Cet_{Sens} HNSCC cell lines and Acq_{Res} variants to cetuximab treatment. As this change was most pronounced in substrates involved in the Akt signaling pathway, we focused further on the interpretation of these results in the next paragraph.

The effects of cetuximab treatment on the phosphorylation of EGFR, Akt and other substrates involved in the Akt pathway were quantified in Cet_{Sens} HNSCC cell lines and Acq_{Res} variants (**Figure 1**). Following cetuximab treatment, activating phosphorylation of EGFR was decreased in all HNSCC cell lines, suggesting inhibition of EGFR signaling. Both Cet_{Sens} HNSCC cell lines (i.e. SC263-S and SCC22b-S) demonstrated decreased phosphorylation of Akt1/2/3 at T308 after cetuximab treatment. T308 is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) in the phosphoinositide 3-kinase (PI3K)/Akt pathway, leading to Akt activation (21). As a result, the PI3K/Akt pathway is inhibited by cetuximab in Cet_{Sens} HNSCC cell lines. In contrast, a considerable increase in Akt1/2/3 phosphorylation at T308 was observed after cetuximab treatment in both Acq_{Res} variants. Furthermore, phosphorylation of Akt1/2/3 at S473, by mammalian target of rapamycin complex 2 (mTORC2) (21), was also enhanced in both Acq_{Res} variants, particularly in SCC22b-R. In addition, cetuximab treatment induced an increased phosphorylation of several downstream substrates of the Akt pathway [e.g. mTOR, p70 S6 kinase, glycogen synthase kinase-3 α/ß (GSK-3α/ß), proline-rich Akt substrate 40kDa (PRAS40) and WNK lysine deficient protein kinase-1 (WNK1)] in Acq_{Res} variants, especially in SCC22b-R. This means that the Akt pathway is still activated in Acq_{Res} cells following cetuximab treatment, leading to anti-apoptotic and pro-proliferative effects.

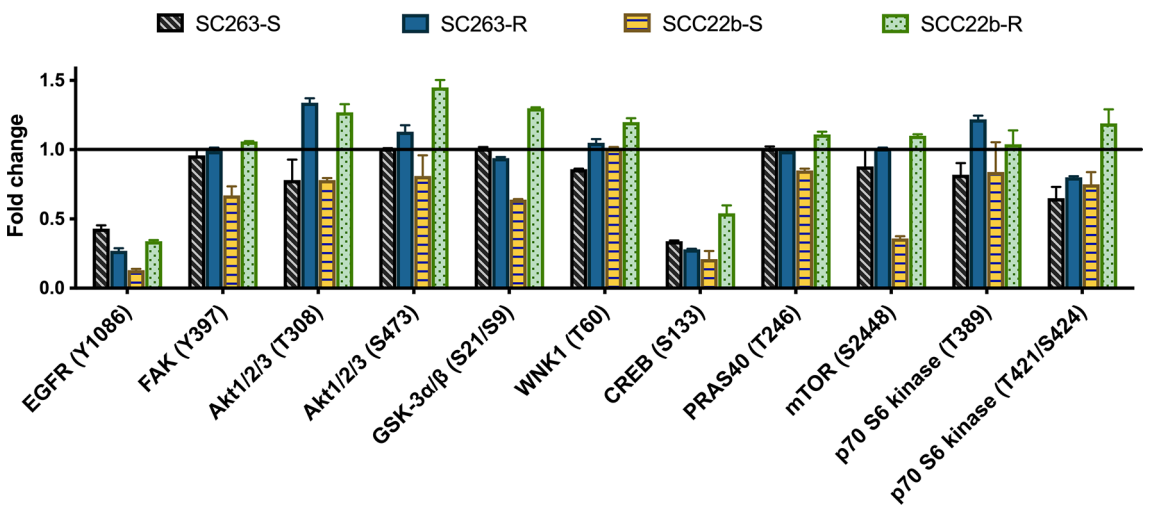

FIGURE 1 | Quantification of the human phospho-kinase array blots of cetuximab sensitive (SC263-S and SCC22b-S) HNSCC cell lines and corresponding acquired cetuximab resistant (SC263-R and SCC22b-R) variants. Graph represents the fold change of selected substrates, i.e. phosphorylated EGFR, Akt1/2/3 and other substrates involved in the Akt pathway, after cetuximab treatment. Protein phosphorylation profiling was conducted with one cell lysate. The fold changes were calculated by the ratio of the mean integrated optical density (duplicates) in treatment versus control groups. Error bars were calculated based on differences between duplicates. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

Western Blot Analysis Confirms Increased Akt1/2/3 Phosphorylation Following Cetuximab Treatment in Acquired Cetuximab Resistant HNSCC Cell Lines

Results from the protein phosphorylation profiling were first validated using western blot. Protein levels of total Akt1/2/3 and phosphorylated Akt1/2/3 at S473 were determined after cetuximab treatment in Cet_{Sens} HNSCC cell lines and Acq_{Res} variants (**Figures 2A, B**). Regarding the expression of total Akt 1/2/3, no change was observed following cetuximab treatment in the SC263-S and the Acq_{Res} SC263-R variant. Cetuximab treatment resulted in a small decrease in total Akt1/2/3 in SCC22b-S, while it resulted in a considerable increase in the Acq_{Res} variant (i.e. SCC22b-R). There was no statistical difference in the change of expression of total Akt1/2/3 following cetuximab treatment across cell lines ($p = 0.130$, **Figure 2B**). Cetuximab treatment did not induce any change in the phosphorylation of Akt1/2/3 at S473 in SC263-S, while a clear decrease in phosphorylated Akt1/2/3 was observed in SCC22b-S after cetuximab treatment. Cetuximab treatment induced a very small increase in the phosphorylation of Akt1/2/3 in SC263-R, while a considerable increase was observed in SCC22b-R. These results suggest a compensatory activation of the PI3K/Akt pathway in reaction to cetuximab treatment in Acq_{res} variants. Statistical analysis showed a significant difference in the change of Akt1/2/3 phosphorylation at S473 after cetuximab treatment between SCC22b-S and SCC22b-R ($p = 0.023$), but not between SC263-S and SC263-R ($p = 1.000$, **Figure 2B**). Comparison of the results of the protein phosphorylation profiling and western blot showed good similarity between both assays. Hence, the increase in Akt1/2/3 phosphorylation after cetuximab treatment in the Acq_{Res} HNSCC cell lines, detected with protein phosphorylation profiling, was confirmed with western blot (**Figure 2C**).

In conclusion, protein phosphorylation profiling demonstrated increased phosphorylation of Akt1/2/3 after cetuximab treatment in the Acq_{Res} HNSCC cell lines. This increase in phosphorylation was confirmed with western blot and was found significantly different between SCC22b-S and SCC22b-R. These results suggest that the combination of cetuximab with an Akt1/2/3 inhibitor might be a potential novel therapeutic combination strategy to overcome acquired cetuximab resistance in HNSCC cell lines.

HNSCC Patients Demonstrate Akt Expression

Before investigating the cytotoxic effect of the combination treatment with the EGFR inhibitor cetuximab and the

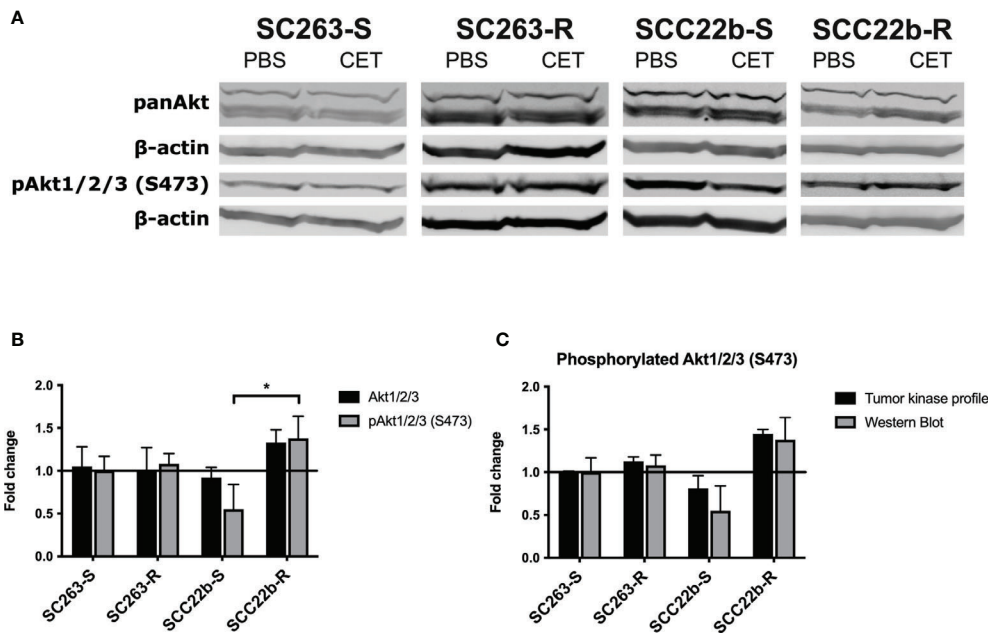

FIGURE 2 | Protein levels of total and phosphorylated Akt1/2/3 in cetuximab sensitive (SC263-S and SCC22b-S) HNSCC cell lines and corresponding acquired cetuximab resistant (SC263-R and SCC22b-R) variants. **(A)** Western blot was used to determine the levels of total and phosphorylated Akt1/2/3 (S473) after cetuximab treatment in cetuximab sensitive and acquired cetuximab resistant HNSCC cell lines. β-actin detection served as loading control. **(B)** Quantification of western blot results for total and phosphorylated Akt1/2/3. Signals were corrected for β-actin and the effect of cetuximab treatment is presented as fold change. Fold changes were calculated by dividing the mean fluorescent signal of the treatment and control groups. The graph represents the average fold change of three independent experiments. **(C)** Fold change of phosphorylated Akt1/2/3 at S473 following cetuximab treatment observed with protein phosphorylation profiling and western blot. CET, cetuximab; pAkt1/2/3, phosphorylated Akt1/2/3; *p-value ≤ 0.050. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

Akt1/2/3 inhibitor MK2206, we determined the expression of Akt isoforms in HNSCC patients using RNA sequencing data from the TCGA dataset (Provisional, RNASeqV2 RSEM, 522 sequenced HNSCC patients). This data demonstrated that there is a significant difference in mRNA expression between Akt1, Akt2 and Akt3 ($p < 0.0001$, **Supplementary Table 2**) in HNSCC patients (**Supplementary Figure 1**). As substantial Akt1, Akt2 and Akt3 mRNA levels were detected in the majority of tumor samples of HNSCC patients, we can conclude that the target of MK2206 is present in HNSCC patients. Therefore, this drug can play a potential role in the treatment of HNSCC patients.

Combining Cetuximab With MK2206 Shows Additive to Synergistic Effects in HNSCC Cell Lines

Possible synergistic effects of treatment with cetuximab and MK2206 were investigated in two Cet_{Sens} HNSCC cell lines and their isogenic Acq_{Res} variants. Two simultaneous combination schedules were evaluated. First, cells were treated simultaneously for 72h with 0-5 μM MK2206 and fixed doses of cetuximab. Fixed doses of cetuximab were based on the outcome of previous monotherapy experiments with total treatment duration of 72h (data not shown). As mentioned above, we previously identified Cet_{Sens} cell lines based on the outcomes of experiments with a total treatment time of 168h (14). After 72h of cetuximab treatment, there was only a limited effect on cell survival. Therefore, we chose two high concentrations of cetuximab (i.e. 25 nM and 50 nM) to use in these simultaneous combination experiments with MK2206. Based on results reported in clinical trials, these concentrations are considered clinically relevant in *in vitro* studies (22). Regarding MK2206, based on early phase clinical trials, clinically relevant concentrations with manageable side effects were estimated at ≤1 μM for *in vitro* studies (23).

The dose-response curves of the Cet_{Sens} HNSCC cell lines and their Acq_{Res} variants are shown in **Figures 3A–D**. A clear concentration-dependent effect of MK2206 after 72 hours of treatment was observed in all HNSCC cell lines. The IC_{50} values for MK2206 monotherapy ranged from 1.752 ± 0.084 μM to 4.764 ± 0.436 μM (**Supplementary Figure 2A** and **Table 1**).

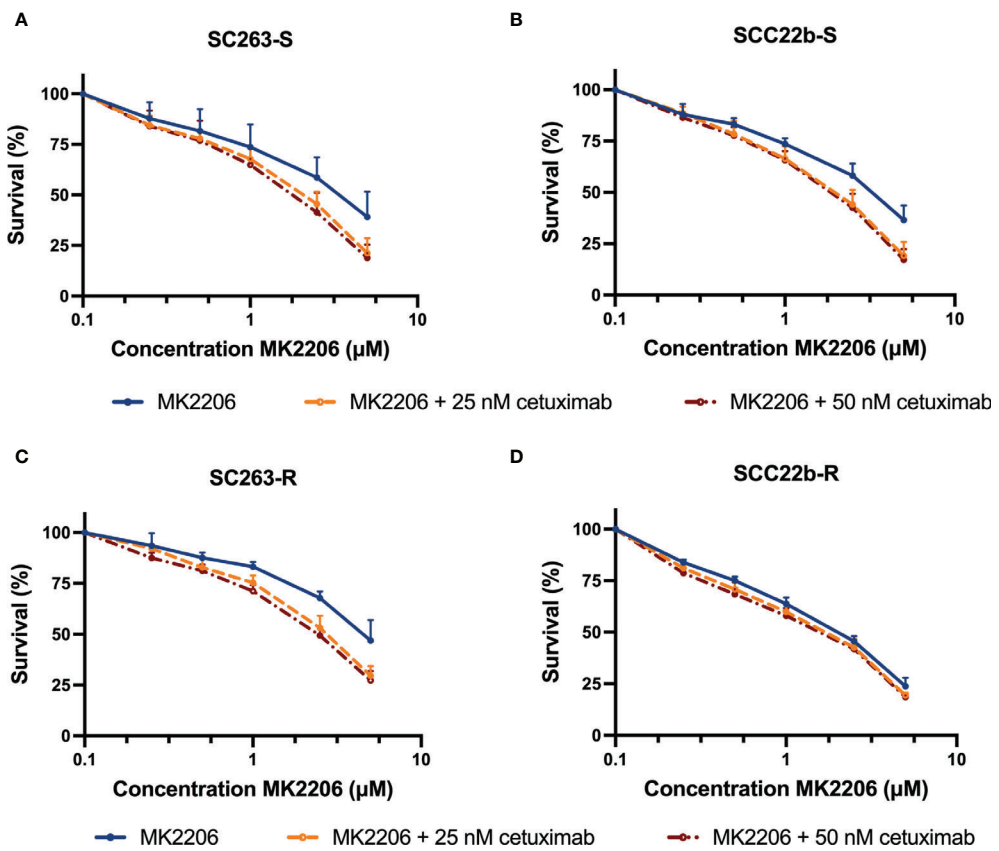

FIGURE 3 | The cytotoxic effect of cetuximab plus MK2206 with a total treatment duration of 72h. Dose-response curves for the cetuximab sensitive HNSCC cell lines SC263-S **(A)** and SCC22b-S **(B)** show a synergistic effect. Regarding the acquired cetuximab resistant variants, dose-response curves indicate a synergistic and additive effect in SC263-R **(C)** and SCC22b-R **(D)**, respectively. Survival curves were corrected for the cytotoxic effect of cetuximab alone. Cells were treated with fixed concentrations of cetuximab, which were chosen based on the outcome of previous monotherapy experiments. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

Acquired cetuximab resistance had no significant influence on the inhibitory potential of MK2206 ($p = 0.954$). Compared to MK2206 treatment alone, simultaneous treatment with cetuximab caused a significant decrease in IC_{50} value ($p \leq 0.048$) in Cet_{Sens} HNSCC cell lines and the Acq_{Res} variants (**Supplementary Figure 2A** and **Table 1**). Furthermore, the CI ranged from 0.707 to 0.917 (**Table 2**). Synergy between cetuximab and 2.5 μM MK2206 was observed in two Cet_{Sens} HNSCC cell lines and one Acq_{Res} variant (i.e. in SC263-S, SC263-R and SCC22b-S with CI ≤ 0.783, **Supplementary Figure 2B** and **Table 2**) after 72h of simultaneous treatment. Combining MK2206 with higher cetuximab concentrations resulted in an increased synergistic or additive effect.

Next, the cytotoxic effect of the second treatment schedule was investigated. Hereby, the cells were treated with fixed doses of cetuximab for 168h and MK2206 (0-2.5 μM) was added during the last 72h of treatment. The goal of this prolonged treatment schedule was to decrease the used drug concentrations. In contrast to the first treatment schedule, Cet_{Sens} HNSCC cell lines were treated with lower concentrations of cetuximab (i.e. 0.5 nM, 1 nM and 5 nM) due to prolonged treatment duration. These fixed concentrations were based on the outcome of previous monotherapy experiments with a total treatment duration of 168h (14).

Figures 4A–D shows the dose-response curves of the Cet_{Sens} HNSCC cell lines and their Acq_{Res} variants. The addition of MK2206 during the last 72h of treatment still resulted in a concentration-dependent cytotoxic effect of this compound. The IC_{50} values for MK2206 ranged from 1.073 ± 0.038 μM to 4.372 ± 1.182 μM (**Supplementary Figures 3A, B** and **Table 3**). Compared to MK2206 treatment alone, simultaneous treatment with cetuximab resulted in a decrease in IC_{50} value ($0.033 \leq p \leq 0.127$). Furthermore, in this treatment regimen, the CI ranged from 0.578 to 0.867 (**Table 4**). Synergy between cetuximab and 1 μM MK2206 was observed in two Cet_{Sens} HNSCC cell lines and one Acq_{Res} variant (i.e. in SC263-S, SC263-R and SCC22b-S with CI ≤ 0.796, **Supplementary Figures 3C, D** and **Table 4**). Combining MK2206 with higher cetuximab concentrations increased the synergistic interaction in Cet_{Sens} cell lines. As this synergistic effect was reached using lower concentrations of both MK2206 and cetuximab, we consider this treatment schedule as the most interesting for further investigation.

DISCUSSION

Targeted therapies are key for the personalized treatment of cancer patients (3). Although treatment with the EGFR-inhibitor cetuximab improves OS in HNSCC patients, therapeutic resistance poses a challenging problem and limits the success of effective anti-EGFR cancer therapies in the clinic (24). Therefore, it is of utmost importance to rationally develop novel combination strategies to overcome this therapy resistance.

Increased or sustained stimulation of EGFR mediates the activation of various signal transduction pathways. Proteins involved in these signaling pathways are potential contributors to the development of acquired resistance to drugs inhibiting EGFR signaling (25). In order to identify the signaling pathways characteristic for acquired cetuximab resistance, protein phosphorylation profiling was performed in HNSCC cell lines. This technique has already been successfully applied in other

TABLE 2 | CI for HNSCC cell lines after treatment with cetuximab plus 2.5 μM MK2206 for 72h.

Cell line	Condition	CI
SC263-S	2.5 μM MK2206 + 25 nM cetuximab	**0.775**
	2.5 μM MK2206 + 50 nM cetuximab	**0.707**
SCC22b-S	2.5 μM MK2206 + 25 nM cetuximab	**0.757**
	2.5 μM MK2206 + 50 nM cetuximab	**0.730**
SC263-R	2.5 μM MK2206 + 25 nM cetuximab	**0.783**
	2.5 μM MK2206 + 50 nM cetuximab	**0.728**
SCC22b-R	2.5 μM MK2206 + 25 nM cetuximab	0.937
	2.5 μM MK2206 + 50 nM cetuximab	0.917

CI < 0.800, CI = 1.000 ± 0.200, and CI > 1.200 indicate synergism, additivity or antagonism, respectively. CI < 0.800 are indicated in bold. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

TABLE 1 | IC_{50} and standard errors for HNSCC cell lines after treatment with MK2206 plus cetuximab for 72h.

Cell line	Condition	IC_{50} MK2206 (μM)	P-value
SC263-S	MK2206	3.311 ± 0.537	–
	MK2206 + 25 nM cetuximab	1.816 ± 0.134	0.068
	MK2206 + 50 nM cetuximab	1.609 ± 0.154	**0.048**
SCC22b-S	MK2206	3.086 ± 0.239	–
	MK2206 + 25 nM cetuximab	1.749 ± 0.120	**0.006**
	MK2206 + 50 nM cetuximab	1.646 ± 0.101	**0.004**
SC263-R	MK2206	4.764 ± 0.436	–
	MK2206 + 25 nM cetuximab	2.545 ± 0.135	**0.015**
	MK2206 + 50 nM cetuximab	2.212 ± 0.109	**0.010**
SCC22b-R	MK2206	1.752 ± 0.084	–
	MK2206 + 25 nM cetuximab	1.446 ± 0.085	**0.020**
	MK2206 + 50 nM cetuximab	1.327 ± 0.082	**0.006**

P < 0.050, significant difference in IC_{50} compared to MK2206 monotherapy. P < 0.050 are indicated in bold. -, cannot be calculated. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

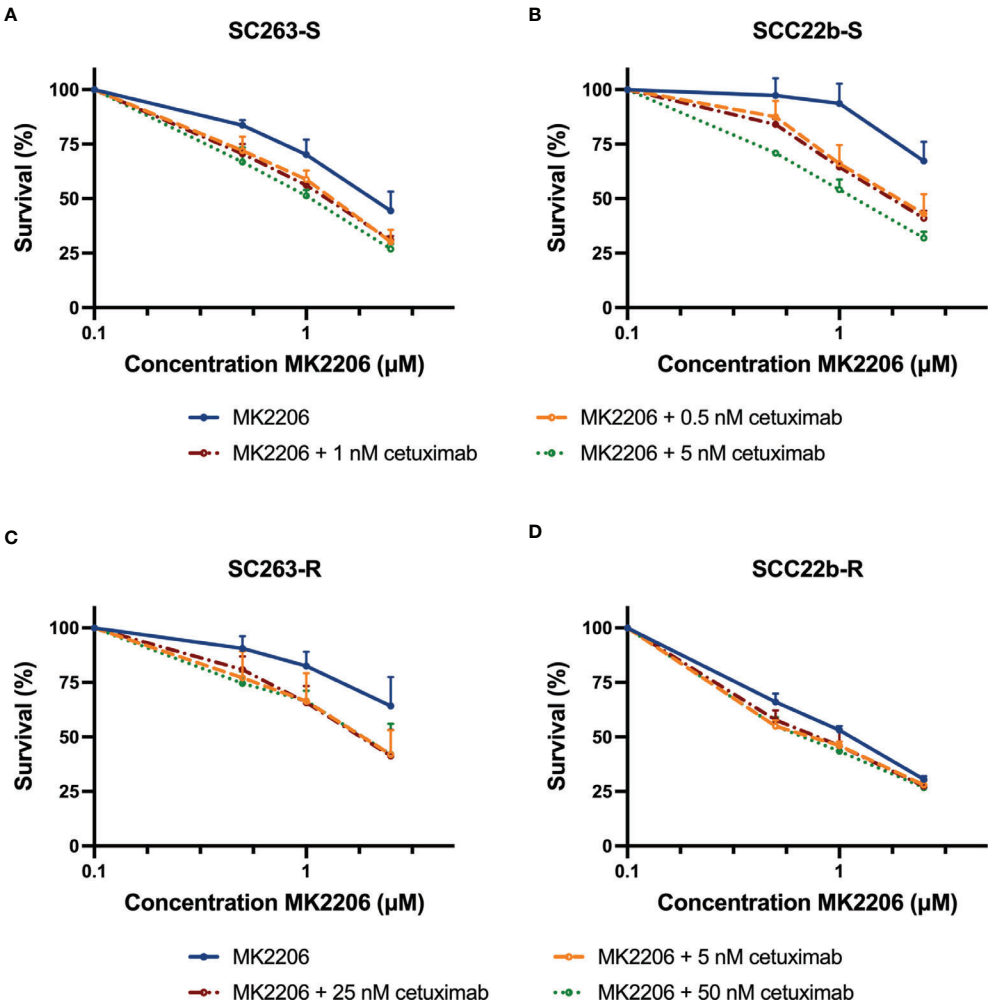

FIGURE 4 | The cytotoxic effect of cetuximab for 168h with MK2206 added during the last 72h of treatment. Dose-response curves for the cetuximab sensitive HNSCC cell lines SC263-S **(A)** and SCC22b-S **(B)** show a synergistic interaction. Regarding the acquired cetuximab resistant variants, dose-response curves indicate a synergistic and additive effect in SC263-R **(C)** and SCC22b-R **(D)**, respectively. Survival curves were corrected for the cytotoxic effect of cetuximab alone. Cells were treated with fixed concentrations of cetuximab, which were based on the outcome of previous monotherapy experiments. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

studies to identify drug resistance mechanisms (26–28). In the present study, the effect of cetuximab treatment on the phosphorylation of several proteins was determined in two Cet_{Sens} HNSCC cell lines and their isogenic Acq_{Res} variants. Based on this protein phosphorylation profiling, novel rationally designed combination strategies were investigated to overcome drug resistance.

Protein phosphorylation profiling showed a differential response of Cet_{Sens} HNSCC cell lines and their Acq_{Res} variants to EGFR inhibition by cetuximab. This profile strongly suggested that increased phosphorylation of Akt1/2/3 following cetuximab treatment is characteristic for Acq_{Res} HNSCC cell lines. In general, Cet_{Sens} HNSCC cell lines demonstrated decreased phosphorylation of EGFR (Y1086), Akt1/2/3 (T308 and S473) and downstream substrates after cetuximab treatment. Although EGFR phosphorylation was decreased, Akt1/2/3 was still phosphorylated and activated following cetuximab treatment in Acq_{Res} HNSCC cell lines. This was supported by the observed increase in phosphorylation of several downstream substrates of Akt, such as mTOR, p70S6 kinase, GSK-3α/β, PRAS40 and WNK1. Thus, in Acq_{Res} HNSCC cell lines, the Akt pathway was still able to exerts its anti-apoptotic and pro-proliferative effects under cetuximab treatment, possibly leading to therapy resistance. Importantly, it is worth mentioning that the number of cell lines used in this study is a limiting factor. Therefore, our data needs to be validated in additional isogenic HNSCC cell lines (and ideally in HNSCC patient samples) in order to strengthen the results. Although further confirmation in

TABLE 3 | IC_{50} and standard errors for HNSCC cell lines after treatment with cetuximab for 168h with MK2206 added during the last 72h of treatment.

Cell line	Condition	IC_{50} MK2206 (μM)	P-value
SC263-S	MK2206	2.067 ± 0.177	–
	MK2206 + 0.5 nM cetuximab	1.253 ± 0.078	0.127
	MK2206 + 1 nM cetuximab	1.200 ± 0.043	0.102
	MK2206 + 5 nM cetuximab	1.002 ± 0.049	0.051
SCC22b-S	MK2206	3.541 ± 0.540	–
	MK2206 + 0.5 nM cetuximab	1.919 ± 0.197	0.112
	MK2206 + 1 nM cetuximab	1.787 ± 0.071	0.082
	MK2206 + 5 nM cetuximab	1.187 ± 0.043	**0.027**
SC263-R	MK2206	4.372 ± 1.182	–
	MK2206 + 5 nM cetuximab	1.851 ± 0.315	**0.045**
	MK2206 + 25 nM cetuximab	1.815 ± 0.201	0.054
	MK2206 + 50 nM cetuximab	1.51 ± 0.264	0.090
SCC22b-R	MK2206	1.073 ± 0.038	–
	MK2206 + 5 nM cetuximab	0.710 ± 0.034	0.052
	MK2206 + 25 nM cetuximab	0.769 ± 0.061	0.102
	MK2206 + 50 nM cetuximab	0.670 ± 0.033	**0.033**

P < 0.050, significant difference in IC_{50} compared to MK2206 monotherapy. P < 0.050 are indicated in bold. -, cannot be calculated. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

TABLE 4 | CI for HNSCC cell lines after treatment with cetuximab for 168h with 1 μM MK2206 added during the last 72h of treatment.

Cell line	Condition	CI
SC263-S	1 μM MK2206 + 0.5 nM cetuximab	0.838
	1 μM MK2206 + 1 nM cetuximab	0.801
	1 μM MK2206 + 5 nM cetuximab	**0.732**
SCC22b-S	1 μM MK2206 + 0.5 nM cetuximab	**0.705**
	1 μM MK2206 + 1 nM cetuximab	**0.688**
	1 μM MK2206 + 5 nM cetuximab	**0.578**
SC263-R	1 μM MK2206 + 5 nM cetuximab	0.805
	1 μM MK2206 + 25 nM cetuximab	**0.796**
	1 μM MK2206 + 50 nM cetuximab	0.806
SCC22b-R	1 μM MK2206 + 5 nM cetuximab	0.867
	1 μM MK2206 + 25 nM cetuximab	0.865
	1 μM MK2206 + 50 nM cetuximab	0.818

CI < 0.800, CI = 1.000 ± 0.200, and CI > 1.200 indicate synergism, additivity or antagonism, respectively. CI < 0.800 are indicated in bold. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

HNSCC cell lines and patient samples is still needed, based on our results, Akt represents a potential target to improve outcome of cetuximab-based treatment in HNSCC patients.

The PI3K/Akt pathway has been shown to regulate various normal biological processes, such as cellular survival, migration, proliferation, differentiation, angiogenesis, protein synthesis and glucose metabolism. Activation of Akt can be initiated by several events, mainly through a receptor-ligand interaction on the cell membrane. This receptor activation results in activation of PI3K, which phosphorylates phosphatidylinositol 3,4-biphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3). The binding of PIP_3 to Akt locates Akt to the plasma membrane and allows its phosphorylation and activation by PDK1. Akt can also be phosphorylated by other substrates and in response to cellular stress, such as ischemia, hypoxia and oxidative stress. The tumor suppressor phosphatase and tensin homolog (PTEN) catalyzes the dephosphorylation of PIP_3 and is the major negative regulator of Akt signaling. Activated Akt exerts its effects by phosphorylating various downstream substrates, all resulting in anti-apoptotic or pro-proliferative effects (29–32). However, the pathway is also associated with a number of oncogenic processes. Indeed, the PI3K/Akt pathway is one of the most frequently dysregulated signaling pathways in cancer, including HNSCC (33, 34).

The present study demonstrates that increased Akt activation (not isoform-specific) is characteristic for acquired cetuximab resistance in HNSCC cell lines. Importantly, this observation needs to be validated in a cohort of HNSCC patient samples in order to elucidate its clinical value. Nevertheless, previous research has already suggested that enhanced Akt activation can play a role in resistance to cetuximab, not only in HNSCC but also in colorectal cancer and non-small cell lung cancer (35–39). Increased activation of the Akt signaling pathway has been associated with genetic alterations in the *PIK3CA* gene (40). For instance, Rebucci et al. have demonstrated that treatment of cetuximab resistant HNSCC cells with cetuximab did not result in the decreased levels of phosphorylated Akt that were seen in cetuximab sensitive HNSCC cells. A mutation in exon 20 of the *PIK3CA* gene, that encodes for the catalytic p110 α subunit of PI3K, was responsible for this persistent Akt activation (36). According to the TCGA dataset, respectively 18.4% and 20.8% of HNSCC patients demonstrate *PIK3CA* mutations or amplification (41, 42). In addition, loss of the tumor suppressor *PTEN* can also lead to persistent activation of the PI3K/Akt pathway (43, 44). According to TCGA, deep and shallow deletions in the *PTEN* gene occur in 3.4% and 24.0% of HNSCC patients, respectively (41, 42). Based on these findings, the genetic background of the HNSCC cell lines used in this study was determined using whole-exome sequencing (data not shown). Both Cet_{Sens} HNSCC cell lines and their Acq_{Res} variants display no mutations, deletions and/or insertions in the *PI3KCA* and *PTEN* genes. As such, the persistent Akt activation in Acq_{Res} HNSCC cell lines following cetuximab treatment cannot be explained by the genetic background of the cell lines. However, genetic alterations in *PIK3CA* and *PTEN*, possibly leading to persistent Akt activation, are present in a significant number of HNSCC patients in the TCGA cohort. Interestingly, both Cet_{Sens} HNSCC cell lines and their Acq_{Res} variants have *TP53* gene mutations that are predicted to have a deleterious effect on protein function. Functional p53 inhibits the PI3K/Akt pathway by regulating the transcription of four genes, which all have an inhibitory effect on Akt and mTOR (45, 46). As a result, mutant p53 can cause sustained activation of the PI3K/Akt pathway. However, as not only Acq_{Res} cell lines, but also Cet_{Sens} cell lines display mutations in *TP53*, the underlying mechanism behind the observed increased phosphorylation of Akt after cetuximab treatment in Acq_{Res} HNSCC cell lines remains unclear. To define the exact role of genetic alterations in the PI3K/Akt pathway, regarding response to cetuximab, more in-depth studies are needed with HNSCC patient samples.

As we found that increased Akt1/2/3 phosphorylation is characteristic for Acq_{Res} HNSCC cell lines and Akt1, Akt2 and

Akt3 are expressed in HNSCC patients, we hypothesized that the combination of cetuximab with an Akt inhibitor might be a potential novel therapeutic strategy to overcome acquired cetuximab resistance. To date, Akt inhibitors are not yet included in clinical practice (32). A phase II study with the pan-Akt inhibitor MK2206 in R/M HNSCC patients showed promising results (i.e. partial responses), but was not moved to phase III so far (NCT01349933). This might be due to the fact that pan-Akt inhibitors targeting all isoforms of Akt have shown to enhance the invasiveness of cancer cells in some cases. In this regard, Brolih et al. demonstrated that Akt1 inhibition leads to a more invasive phenotype in HNSCC tumors that primarily express Akt1 (47). Consistently, the latter was also observed in several studies for breast cancer (48–51). This suggests that the expression of specific Akt isoforms can influence the outcome of pharmacological Akt inhibition. Therefore, Akt isoform analysis may be necessary in order to predict the outcome of pan-Akt inhibitors (47). Alternatively, the use of isoform-selective Akt inhibitors, which have recently been developed (52), may also offer a solution to overcome the potential limitations of the pan-Akt inhibitor MK2206. On the other hand, it has been suggested that future studies should explore mechanism-based combination strategies with chemotherapy or other molecular targeted agents (53).

In the present study, our results provide a rationale to combine cetuximab with MK2206 to overcome acquired cetuximab resistance in HNSCC. To test our hypothesis, we examined the effects of treatment with cetuximab and the Akt inhibitor MK2206 in Cet_{Sens} HNSCC cell lines and Acq_{Res} variants. Hereby, two simultaneous combination schedules were tested. Synergy between cetuximab and MK2206 was observed in two Cet_{Sens} HNSCC cell lines and one Acq_{Res} variant in both simultaneous treatment schedules. An additive effect was observed in the Acq_{Res} SCC22b-R HNSCC cell line. Interestingly, MK2206 monotherapy demonstrated already a large cytotoxic effect in the SCC22b-R cell line compared to the other three cell lines used in this study. A possible explanation is that the SCC22b-R cell line demonstrated the largest increase in phosphorylation of Akt1/2/3 in the protein phosphorylation profiling analysis. As a result, the increased cytotoxic effect of MK2206 treatment alone might explain the observed additive interaction with cetuximab. Additional research is required to further elucidate the molecular mechanisms underlying the additive to synergistic effect between cetuximab and MK2206. Overall, this study demonstrated the potential of Akt inhibition in combination treatments to overcome acquired cetuximab resistance in HNSCC.

Besides the rational design of novel combination strategies, characterization of cetuximab resistance mechanisms can also lead to the identification of predictive biomarkers. To date, no definitive biomarkers have been identified to predict the efficacy of EGFR-targeting agents in patients with HNSCC (25, 54). We found in literature that phosphorylation of Akt at S473 serves as an independent prognostic marker for radiosensitivity in advanced HNSCC and that inhibition of Akt phosphorylation with pharmacological compounds might circumvent resistance to radiotherapy (55). Moreover, it has already been reported that phosphorylated Akt might be a potential predictive biomarker for EGFR-targeted therapies. For instance, lower phosphorylated Akt was observed in cetuximab sensitive HNSCC tumors in cell line xenograft models (56). Furthermore, Lyuo et al. analyzed a cohort with 50 oral squamous cell carcinoma patients who received cetuximab-based induction chemotherapy and found that lower expression of phosphorylated Akt was associated with better disease-free survival (57). In addition, the ECOG2303 phase II trial showed that biomarker signatures consistent with activation of the PI3K/Akt pathway are associated with inferior outcomes to cetuximab-containing chemoradiotherapy regimen (37). These results encourage additional research to precisely define the role of the PI3K/Akt pathway as predictive biomarker for EGFR-targeting agents in HNSCC.

CONCLUSION

In conclusion, protein phosphorylation analysis demonstrated that increased Akt1/2/3 phosphorylation is characteristic for acquired cetuximab resistance in HNSCC cell lines. Furthermore, we observed an additive to synergistic interaction between the EGFR inhibitor cetuximab and the pan-Akt inhibitor MK2206 in cetuximab sensitive and acquired cetuximab resistant HNSCC cell lines. Overall, these data support the hypothesis that downstream effectors of the PI3K/Akt pathway serve as promising drug targets in the search for novel therapeutic combination strategies that are able to overcome resistance to anti-EGFR treatment in HNSCC patients.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, HZ, IDP, JBV, and AW. Data curation, IDP and HZ. Formal analysis, IDP and HZ. Funding acquisition, MP, FL, and AW. Investigation, IDP, HZ, and HB. Methodology, IDP and AW. Project administration, IDP, HZ, and AW. Resources, PP, FL, and AW. Supervision, IDP and AW. Visualization, IDP, HZ, and AW. Writing—original draft, IDP, and HZ. Writing—review & editing, HZ, IDP, HB, MP, JBV, FL, and AW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.697967/full#supplementary-material

Supplementary Figure 1 | mRNA expression level of Akt1/2/3 in HNSCC patients, available from TCGA. The graph shows the log transformed mRNA expression (mean and standard deviation) of Akt1/2/3 from 522 HNSCC patients (individual dots). This dataset (TCGA Provisional, RNASeqV2) was downloaded from cBioportal. *, p-value ≤ 0.050.

Supplementary Figure 2 | The cytotoxic effect of cetuximab plus MK2206 with a total treatment duration of 72h. **(A)** IC_{50} of MK2206 for HNSCC cell lines after treatment with MK2206 alone and in combination with cetuximab. **(B)** Combination index (CI) versus fraction affected (FA) plot of 2.5 μM MK2206 with fixed doses of cetuximab (25 nM and 50 nM) in SC263-S (red), SCC22b-S (blue), SC263-R (green) and SCC22b-R (orange). *, significant difference in IC_{50} compared to MK2206 monotherapy ($p < 0.050$). CI < 0.800, CI = 1.000 ± 0.200 and CI > 1.200 indicated synergism, additive effect and antagonism, respectively. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

Supplementary Figure 3 | The cytotoxic effect of cetuximab for 168h with MK2206 added during the last 72h of treatment. **(A)** IC_{50} of MK2206 for cetuximab sensitive HNSCC cell lines after combination treatment. **(B)** IC_{50} of MK2206 for acquired cetuximab sensitive HNSCC cell lines after combination treatment. **(C)** Combination index (CI) versus fraction affected (FA) plot of 1 μM MK2206 with fixed doses of cetuximab (0.5 nM, 1 nM and 5 nM) in SC263-S (red) and SCC22b-S (blue). **(D)** Combination index (CI) versus fraction affected (FA) plot of 1 μM MK2206 with fixed doses of cetuximab (5 nM, 25 nM and 50 nM) in SC263-R (green) and SCC22b-R (orange). *, significant difference in IC_{50} compared to MK2206 monotherapy ($p < 0.050$). CI < 0.800, CI = 1.000 ± 0.200 and CI > 1.200 indicated synergism, additive effect, and antagonism, respectively. Suffix -S: cetuximab sensitive cell line and suffix -R: acquired cetuximab resistant cell line.

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Rehabilitation Needs of Head and Neck Cancer Patients and Stakeholders: Case Study

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Background: The incidents of Head and Neck Cancer (HNC) are rising worldwide, suggesting that this type of cancer is becoming more common. The foreseen growth of incidents signifies that future rehabilitation services will have to meet the needs of a wider population.

Objective: The aim of this paper is to explore the needs of patients, caregivers and healthcare professionals during HNC rehabilitation.

Methods: This paper reports the empirical findings from a case study that was conducted in a cancer rehabilitation center in Copenhagen to elicit the needs of HNC cancer patients, informal caregivers and healthcare professionals.

Results: Four areas of needs during the rehabilitation process were identified: service delivery, emotional, social and physical needs. Service delivery needs and emotional needs have been identified as the most prevalent.

Conclusions: Stakeholders' needs during the rehabilitation process were found to be interrelated. All stakeholders faced service delivery challenges in the form of provision and distribution of information, including responsibilities allocation between municipalities, hospitals and rehabilitation services. Emotional and social needs have been reported by HNC patients and informal caregivers, underlining the importance of inclusion of all actors in the design of future healthcare interventions. Connected Health (CH) solutions could be valuable in provision and distribution of information.

Keywords: connected health (CH), head and neck cancer, stakeholders, informal caregivers, rehabilitation

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INTRODUCTION

In 2016, a milestone has been reached and for the first time Head and Neck Cancer (HNC) was acknowledged to resemble a chronic illness (1). HNC has been recognized as one of the few cancer types that requires anticipatory treatment and prolonged rehabilitation (2). This can be related to HNC survivorship experience, which is accompanied with high morbidity and reduction in patients' quality of life. Therefore, the main aim of rehabilitation of HNC is to improve quality of life, which

Abbreviations: HNC, Head and Neck Cancer; CKSK, Center for Kræft og Sundhed København; GPs, General Practitioners.

is defined by the WHO "as complete physical, mental and social welfare state and not only the absence of the disease" (3). HNC cancer invades both physical and emotional aspects of life causing often facial disfigurements, disrupting the pleasure of eating (taste, smell), compromising respiratory control, sneezing and laughing mechanisms (1).

Dysphagia is the most common long-term side-effect of chemoradiotherapy (4), and is related to the difficulty of swallowing and movement of food through the 'throat' or pharynx (5). Dysphagia is associated among others with prolonged tube feeding and fundamental changes to eating patterns, social activities and consequently poorer quality of life (6, 7). Apart from the physical side-effects patients often experience psychological disorders. Newly diagnosed HNC patients may experience adjustment disorders and major depression (8). Emotional numbing has been reported as a factor that can increase further patients' distress, as sharing feelings with their beloved ones was found to be a challenge on its own (9).

HNC incidence has also implications on the patient's family and friends. Patients' relatives and friends, known as informal caregivers, provide support to patients in the treatment and post-treatment periods (10). Caregiving is a demanding and challenging assignment. Informal caregivers of HNC patients may experience even higher levels of anxiety than patients within the six month interval following diagnosis, a state which is often related to the fear of cancer recurrence (10). Caregivers' psychological health depends on various factors, such as patients' disease severity, but also on their personality and resources (11). The essential role of informal caregivers and the importance of their wellbeing, has led to suggestions for specialized psychosocial interventions. These interventions have been seen as an effective approach to alleviate informal caregivers' psychosocial burden, while also having a positive impact on the quality of patients' lives (12).

Despite the fact that HNC patients have multiple and severe unmet needs compared to other cancer types (13) and that rehabilitation has a significant role in addressing those needs (14–16), rehabilitation is not always part of the clinical practice in HNC care (17).

The Danish healthcare system can be characterized by decentralized administration, distributed to 5 districts and 98 municipalities. As seen in **Figure 1** below, it operates in three levels; the National (Ministry of Health), the Regional (5 regions), the Local level (98 Municipalities). The Danish healthcare may be unique in its care delivery as it allocates the rehabilitation responsibilities between Hospitals and Municipalities. However, in aspects such as healthcare quality and patient safety it is similar to other European systems (18). In addition to emergency treatments, the hospitals also provide rehabilitation services to patients with cancer, while municipalities play a key role in the prevention of the disease (19, 20). Rehabilitation of cancer patients includes a variety of interventions, such as physical therapy, psychosocial support and physical training (21–23). Cancer rehabilitation programs in Danish municipalities have been available since 2007. Municipalities are only responsible for the generalized rehabilitation interventions (24, 25), while the hospitals hold the main responsibility for specialized rehabilitation services for patients with cancer (26).

Previous studies have focused on addressing the rehabilitation needs of HNC patients and stakeholders. Healthcare professionals have reported cases in which lack of expertise to perform medical procedures and gaps in service provision led to poor services related to psychosocial needs of patients and caregivers (27, 28). The unmet information needs have been also discussed from the perspectives of patients and caregivers. Caregivers reported lack of information associated with diagnosis and treatment phases. Studies concluded that patients and caregivers also require more information about self-care, pain and distress management, while the preferable mode of information provision was from healthcare professionals and digital interventions (29). Self-management interventions have been proposed as solutions to facilitate care for patients and caregivers (30). The study of Bard et al. points out that some of the patients and caregivers' needs were interrelated, while peer to peer social support was found to be important (30). Ringash et al. investigated the physical, emotional and cognitive needs of HNC patients and caregivers, concluding that 60% to 70% of the stakeholders reported unmet needs (31). A literature review confirmed that further investigation related to the informational and support needs of stakeholders is necessary, addressing a communication gap between patients, caregivers and healthcare professionals (32). The study of McEwen et al. utilized focus groups to address the needs of patients, caregivers and healthcare professionals, to provide insights pertinent to facilitators and barriers to recuperate functional health (17). While they found a significant amount of interrelated needs between the three stakeholders, healthcare professionals have been acknowledged to have many specific needs compared to the other two groups.

The findings above demonstrate that patients and stakeholders face a number of unmet needs in the course of HNC rehabilitation. A relation between unmet patients' needs and caregiving burdens suggests that interventions should focus their efforts on both stakeholders (33). However, the "equation" of rehabilitation provision also includes the healthcare professionals. The present study reflects upon the studies of (17, 32, 33) by exploring the needs of patients, caregivers and healthcare professionals during HNC rehabilitation. We address the following research question: *What are the needs of the different stakeholders during Head and Neck cancer rehabilitation in the Danish context?* We explored this research question by analyzing a case study that was conducted at the Center for Kræft og Sundhed København (CKSK), during a service design course for a Master's degree. CKSK is the largest and newest rehabilitation center in Denmark that offers rehabilitation services to approximately 1,500 cancer survivors every year. Our case study explores the phenomenon of interrelated needs and the possibility to develop Connected Health (CH) rehabilitation solutions through collaboration of HNC patients, family members and healthcare professionals.

Connected Health (CH) is a conceptual model for health management (usually *via* mobile, wireless, telehealth interventions) (34) where devices and services are designed around patient's needs, and health data is shared, in such a way

FIGURE 1 | The Danish healthcare system, an overview of responsibilities between the 3 levels (National, Regional and Local) (18).

that patients are able to receive proactive and efficient care (34). CH interventions can enhance the quality of life of patients during cancer care, focusing not only on personalized solutions, but also on multidisciplinary and inclusive approaches for rehabilitation (35).

METHODS

Case Study in CKSK Rehabilitation Center

We conducted a single-case study in the second half of 2017, where the unit of analysis was the CKSK head and neck cancer rehabilitation center. According to Yin, case study is an empirical inquiry that explores a contemporary phenomenon within its real-life context utilizing multiple sources of evidence in order to understand and explore the phenomenon under investigation (36). While most of the data collection was done in the context of Center for Kræft og Sundhed København (CKSK), supplementing data was also collected through an organization called Sundhed.dk. Sundhed.dk is a national and publicly funded health data and information portal in Denmark, complementing the service delivery needs from the viewpoint of a provider of digital solutions for rehabilitation service.

The selection of the case partners for this study reflected upon a need. Only a few municipalities in the Danish district offer rehabilitation programs for HNC patients, therefore CKSK center has to share professional expertise and knowledge with other municipalities that lack rehabilitation expertise. This problem has inspired CKSK and Sundhed.dk to collaborate in order to create a digitally assisted solution that can overcome this problem and share rehabilitation expertise and knowledge to other parts of Denmark. Before initiating the project, the case partners wanted to explore and fully comprehend the needs of the stakeholders to develop a CH solution which would serve its purpose. **Figure 2** presents a simplified version of a cancer patient's pathway in Denmark.

Data Collection

Data collection was performed by five groups of service design students (24 individuals), who were instructed and supervised by three service design researchers from design, engineering and information systems disciplines (one of the supervisors is a co-author of this paper). The 24 students who participated in the course were divided into five groups. Each group selected one of its members to conduct the interviews. As the goal of the case study was to collect rich, detailed information about the needs, the data collection was qualitative in nature.

The stakeholder participants were selected, as a convenience sample, by the center. The selection was based upon the following three criteria: (1) patients in their first year of rehabilitation; (2) diversity of roles of healthcare professionals; (3) willingness and availability of stakeholders to participate in relatively lengthy interviews in an active way. Data collection and understanding of the HNC domain included two phases: presentations from healthcare professionals and written material, and semi-structured interviews.

Phase 1: Presentations From Healthcare Professionals and Written Material

Written material about HNC rehabilitation was made available to the students to understand the basic concepts, and the rehabilitation center management and nurses introduced their subjective views on problems and needs. In addition, a management representative and design professional from Sundhed.dk presented their views on possibilities and challenges of digital service delivery for cancer rehabilitation. Following this, a session with rehabilitation professionals was arranged to collect data about the needs of different areas of rehabilitation. Healthcare professionals, namely an occupational therapist, a physiotherapist, a social worker, a dietician and a nurse, gave short presentations to the groups describing their work practices and relationships to patients, as well as their specific needs in regards to their occupation. By identifying the different stakeholders and the interplay between them, the student groups were able to characterize their roles and values pertinent to the rehabilitation process (37).

Phase 2: Semi-Structured Interviews

Four HNC patients, five healthcare professionals and four informal caregivers were interviewed face-to- face by all the groups of students at the premises of the center. Participants were advised to express and elaborate on their personal

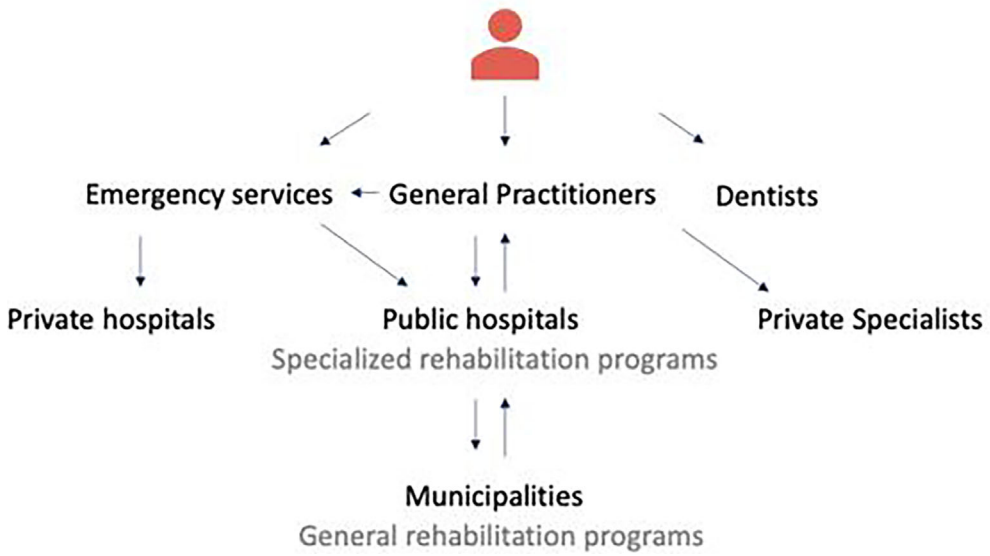

FIGURE 2 | A simplified version of a cancer patient's journey in the Danish healthcare system. The care process starts with GP's who are the gatekeepers of the secondary care, then moves to Hospitals for provision of medical treatment and specialized rehabilitation and finally to Municipalities for generalized rehabilitation services. Dentists are self-referred specialists.

experiences during their rehabilitation and encouraged to comment about the content of questions, if they felt they were too personal or made them feel uncomfortable. Semi-structured interviews were organized around themes such as rehabilitation, communication, network, and close relations, with the aim to gain knowledge about the stakeholders needs and requirements. Students performed the semi-structured interviews with the different stakeholders. The interview questions were developed together with their supervisors to gain knowledge about their needs and elicit requirements for proposing future CH rehabilitation interventions. Students audio recorded the interviews and transcribed them verbatim, applying thematic analysis methods (38). Semi-structured interviews with the different stakeholders lasted between 30 minutes to 1 hour, depending on the preference of the stakeholder. All patients were interviewed in Danish (see example questions in Multimedia Appendix 1). To explore the rehabilitation services provided in municipal districts other than Copenhagen, one of the student groups conducted phone interviews with cancer coordinators from four municipalities of different sizes across Denmark. Desk research, also known as secondary research, was also used by all the groups to collect information about the relevant stakeholders and to acquire general technical and medical knowledge concerning patients with HNC (39). The students were in their final year of their master degree and were sufficiently trained to utilize the aforementioned methods as they have received specialized courses to attain this knowledge. Data collection methods were developed and implemented under the supervision of their teachers and teaching assistants. **Figure 3** presents an overview of the data analysis process from the viewpoints of the students and authors.

Participants' Characteristics

Four HNC patients during their rehabilitation were recruited from CKSK. Three males and one female patient, age ranged from 34 to 74, in their first year of rehabilitation provided consent to participate in this study. Patients during their first year of rehabilitation were chosen to be included, as that is the phase they have frequent interactions with the rehabilitation service. Patients with severe challenges in communication were excluded from the study. Inclusion/exclusion criteria was made by the healthcare professionals of the rehabilitation center. **Table 1** presents the patients characteristics.

Four informal caregivers and five healthcare professionals were also recruited. The informal caregivers were three females (spouses of the patients) and one male (father of the patient). The Healthcare professionals who participated in the study were an occupational therapist, a physiotherapist, a social worker, a dietician and a nurse, who was acting as a contact person for the patients. To explore the rehabilitation services provided in municipal districts other than Copenhagen, one of the groups also conducted phone interviews with cancer coordinators from four municipalities of different sizes across Denmark.

Data Analysis

For the purpose of this paper, a meta-synthesis of the qualitative data was conducted by the authors to abstract and generalize through the process of translation and synthesis (40, 41) - to thin out (42) - from the thick descriptions generated by the 5 groups of students (n=24). This was not a process of secondary data analysis of the primary data collected by the groups (e.g. recordings of group discussions, or transcripts of interviews), but an analysis of thick descriptions generated by the groups during the data collection

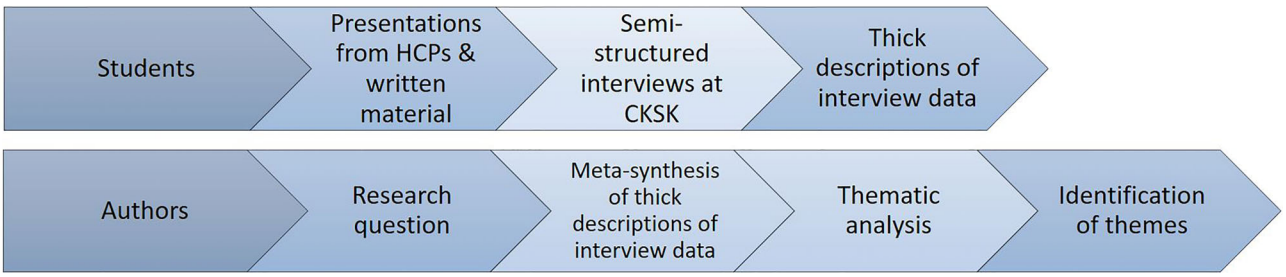

FIGURE 3 | Overview of the data analysis process from the perspectives of the students and authors.

TABLE 1 | HNC patients' characteristics.

ID	Participant #1	Participant #2	Participant #3	Participant #4
Gender	Male	Male	Male	Female
Age	74	67	34	56
Education[a]	BA[b]	MA[c]	MA	HD[d]
Employment[e]	No	Yes	Yes	No
Living condition	Single	With family	With family	Single
Rehab. time	9 months	12 months	3 months	10 months
HNC type	N/D[f]	N/D	Salivary glands	HPV[g]
Treatment	Surgery 33 x radiation	Surgery 35 x radiation 3 x chemotherapy	Surgery 35 x radiation	35 x radiation 2 x chemotherapy

[a]Education completed, [b]Bachelor's Degree, [c]Master's Degree, [d]Higher Degree, [e]Employment status, [f]Not Defined, [g]Human papillomavirus.

process. The meta-synthesis was done through a thematic analysis, where the thick descriptions created by the data collection groups were analyzed to identify themes and sub-themes (43) that would be descriptive for identifying and understanding the needs of stakeholders. "Thick description refers to the researcher's task of both describing and interpreting observed social action (or behavior) within its particular context" (39). Coding was used as an interpretive instrument for dealing with the content (44). We used the resulting thick description as an articulation of how we jointly see and understand the phenomenon we were studying, intertwining it with the analysis process for not merely including detail, but also interpreting and translating the detailed description of observations in the social, physical, organizational, technical and physiological context (45). All the four authors read and reviewed the thick descriptions created by the data collection groups. The authors adopted a collaborative process to analyze the thick descriptions, which was performed in two phases. Initially, the first two authors had two sessions in which the findings were discussed and themes and sub-themes were defined. In the second phase, the third and fourth authors were involved in the data analysis process to validate and discuss the justifications and choices done. After a briefing by the first author, all the authors had an additional session, in which a discussion on the themes took place and the data were grouped into the final themes and sub-themes.

Ethical Considerations

The ethics committee of the IT-University of Copenhagen follows the principles of the Danish Code of Conduct for Research Integrity (46). An email approval for the use of data collected by the students and the analysis and interpretation work they did was asked before meta-analysis. The ethics approval process of the Center for Kræft og Sundhed København was followed in approaching and working with patients and care professionals. The participants were presented an informed consent form which they signed prior to the study, they were provided contact information of a contact person, and they were informed regarding their right to withdraw from the study at any time.

RESULTS

The findings below are presented as follows: (1) HNC patients' needs, (2) Informal caregivers' needs and (3) Healthcare professionals' needs. The following four sub-themes have been identified: service delivery needs, emotional needs, physical needs and social needs. **Table 2** provides a summary of the results.

HNC Patients' Needs

Patients' rehabilitation needs are unique, as the physical and emotional symptoms are comprehensive and diverse. Four areas of needs have been identified: service delivery, emotional, social and physical.

Service Delivery Needs

Lack of information and organizational challenges were reported by the patients. More specifically, they addressed lack of

TABLE 2 | Summary of needs - HNC patients, informal caregivers and healthcare professionals.

Service Delivery needs	Emotional needs	Social needs	Physical needs
HNC Patients			
Lack of information and organizational challenges Dissatisfaction caused by paper format Information management Tight rehabilitation schedule	Need for emotional support Support fuels HNC Patients motivation Need for a disease free space	Need for socializing with people they identify with Socialization fuels their motivation	Need for practical suggestions relating to nutrition and eating difficulties
Informal Caregivers			
Lack of information Information management	Need for support in dealing with their anxiety, stress	Need for support in dealing with their anxiety and feelings of guilt and shame	N/A
Healthcare Professionals			
Lack of contextual knowledge, experience and reliable information Organizational and structural challenges Need for technological support to share knowledge between municipalities	N/A	N/A	N/A[a]

[a]*Not applicable.*

information at hospitals concerning the rehabilitation services provided by the municipalities.

A considerable 'pain point' for patients was related to unanswered questions. They felt that they do not have the opportunity to receive answers to questions that may suddenly arise.

The findings highlighted issues arising from the service provision. Patients were dissatisfied receiving information regarding the rehabilitation process mostly on paper:

"Yes, they could come up with something better. Something people can see on a screen, you know? That would help me, definitely."

Another point of contention was related to information management. It was hard for patients to keep track of all the information and translate it to relevant knowledge and actions, especially during the cancer diagnosis phase:

"[…] it is insanely hard before the treatment period to understand what is going to happen. So really really hard. Hmm, and there was very much information overload."

The experience of another patient concerning the management of medical information during the diagnosis period was the following:

"I could not tell them [the parents] anything because I did not have the information. I was totally far away when I was given the information."

These findings indicate how important it is for patients to receive information in a comprehensive way in order to have an overview of their cancer treatment and rehabilitation process. The majority of them found it demanding and difficult to manage and keep control of their tight treatment schedule.

Emotional Needs

Alongside with the physical support, patients highlighted the paramount importance of communication and psychological support from relatives, friends and healthcare professionals throughout the treatment and post-treatment periods:

"[…] find that person, who can coach you. It does not need to be a coach […]. But find that person, who can help you to take care of your mind."

Some of the patients underwent the rehabilitation process without having direct support from relatives or friends. They praised the experience of rehabilitation at CSCK and explained how crucial was the emotional support they received from healthcare professionals and other cancer patients.

The emotional support could trigger motivation. The need for motivation and sense of duty to participate actively in the rehabilitation process has been addressed by the HNC patients. A concrete example is pertinent to physical activities, as patients mentioned that they have to perform exercises regularly, which required a lot of discipline especially after the end of the rehabilitation program and when they were outside the center:

"My sister has completed the exercises alongside me every day when I stayed with her. It became a habit."

They argued that the provision of emotional support is also essential as the nature of the detrimental side-effects of HNC require long-term efforts to appreciate their progress. Struggling to monitor their own progress was reported to be a demotivating factor, especially if they do not have insight into their own progress.

Finally, HNC patients expressed a demand for a disease-free space. They addressed the need to hold conversations about everyday life out of the cancer context, as they felt that they were constantly being reminded of their medical condition:

"I would rather talk about anything else [than my disease] […] I had a hard time talking to my regular friends about this. Because I didn't want to be "the sick one". I didn't want to talk about my cancer."

This could pinpoint that when patients are engaging in their pre-cancer environment they desire to be again their disease-free selves.

HNC patients valued the support from relatives and family as an essential factor that had a positive impact on their life. They argued that informal caregivers supported them in coping with the burden that the disease posed and to manage their stress levels:

"My mother and wife were completely destroyed. My father also for that matter - and my brother. The whole family. It was a huge shock."

A contact person in the rehabilitation center was considered to be a key individual. The contact person utilizes informal conversations, specialist knowledge and emotional support to customize the rehabilitation program to the patient's specific needs:

"I've always been able to call her [my contact person] at any point in time - if I've had a bad episode - and talk to her. She has been there 100% of the time. […] And if I couldn't get a hold of her, she has always called me back […]."

HNC patients asserted that the contact person was highly appreciated and acted as a motivation trigger for the patients.

Social Needs

Apart from emotional needs, patients addressed a need for social interaction with other HNC patients. Socializing with patients that faced similar life-threatening disease considered to be essential for patients with HNC:

"[…] we talked during breaks at the training session, while waiting on the [workout] machines […] and we talked about "what have you been operated for?" and "what have you experienced". It was pretty comforting to get to know each other, and it encouraged me to go exercising."

Socializing with other HNC patients enabled them to communicate their concerns pertinent to treatment and their rehabilitation challenges, including common side- effects of radiation therapy, such as sore mouth and swallowing problems. Patients argued that social support is an important part of their rehabilitation and act as a motivator to perform swallowing exercises on a regular basis:

"It has been helpful to have someone there to get me to do them [exercises]. If I had only received papers for the exercises I wouldn't have done them."

The demand for social support found to be higher in areas outside the capital where there is lower concentration of HNC cancer patients and lack of specialized rehabilitation centers.

Nevertheless, a few patients reported as a barrier to social support the lack of identification with other cancer patients due to issues such as age gap. A patient expressed the need to identify himself with other patients in a similar age group. Since he could not find any groups/events he felt comfortable in, he searched for videos on YouTube to learn from and identify with patients more resembling his own life situation and/or age group.

Physical Needs

The majority of patients reported eating challenges after treatments as a side-effect of radiation therapy. Effects such as dental problems, swallowing difficulties and taste changes, along with pain and fear associated with food consumption:

"It's not that smart, you cannot eat in public. Sometimes you swallow it down the wrong pipe. Then grab a napkin and stand and cough it up at a speed of hell. It's not that smart to eat in public."

Eating challenges faced by patients are typically long-term. They start from the beginning of the medical treatment, lasting long after the end of the rehabilitation period:

"[…] I wish it was recommended to me from the beginning, as I later learnt, that many people appreciate blueberries before and after treatment."

Informal Caregivers' Needs

The needs of informal caregivers of HNC patients were pertinent to the patients' mental health and to the practical aspects of everyday life.

Service Delivery Needs

In terms of practical information, informal caregivers argued that there were times they felt that they missed the knowledge of how to accommodate the patients' needs in order to establish a supportive relationship during the rehabilitation process. In addition, they experienced distress due to lack of information about the available rehabilitation options at hospital, as well as during the rehabilitation process. For example, one of the common side-effects of the radiation therapy is tooth decay, nevertheless, they argued that they were not aware that dental treatment is not part of the rehabilitation process. Another challenge that was identified related to food preparation and oral exercises. Food preparation for HNC is particularly challenging due to their new nutritional needs after the radiation therapy. Side-effects such as swallowing disorders and pain posed challenges to food preparation and required the adoption of a specific diet.

Emotional Needs

The majority of informal caregivers often experienced stress and anxiety, which negatively affected their mental health in daily life. They experienced difficulties in expressing their feelings in the context of cancer. More specifically, relatives revealed that feelings of guilt and shame often remained unspoken, while taboo subjects such as sexual intercourse were neglected.

Social Needs

Social needs were related to the emotional support of HNC patients. The informal caregivers expressed the desire to speak and connect with someone that understands their point of view without criticizing them:

"You're left with forbidden feelings. Things you do not like to say out loud."

Healthcare Professionals' Needs

Healthcare professionals' needs were also acknowledged, as their input was considered valuable in having a holistic overview of the service ecology. The findings represent different healthcare professional specialties views relevant to HNC rehabilitation and were focused on service delivery needs.

Service Delivery Needs

Healthcare professionals who were located outside the capital area stated that they lack contextual knowledge, experience and information pertinent to HNC:

"In other municipalities they need concrete knowledge about what to do with this type of patients […] because they don't have the same opportunities."

The majority of them did not have previous experience and knowledge in treating HNC patients, indicating that these cases require specialized interventions. A desire for reliable information among healthcare professionals highlighted the potential value of interventions which are reliable, trustworthy and accurate. In some cases, healthcare professionals from municipalities in Jutland had to arrange phone consultations with CKSK to find answers to their questions:

"How do we train the musculature?", "What kind of exercises should the physiotherapist use?"

Healthcare professionals agreed that digital solutions for knowledge sharing could have a positive impact on their performance. When healthcare professionals sought knowledge the main source of information was their colleagues. If their colleagues were not available, then they would seek knowledge through Sundhed.dk, Promedicin.dk or other sources of information. In the same vein, the value of knowledge and experience sharing between patients highlighted by healthcare professionals as a means that would facilitate their work. However, healthcare professionals mentioned that it is challenging to convince patients to gather and share their experiences in a formal setting.

While knowledge sharing is a common practice in the secondary sector, healthcare professionals highlighted that knowledge sharing practices are not common in the primary sector:

"In the secondary sector we shared a lot of data, insights and discussed a lot across different departments. In the primary care there is no such thing as knowledge sharing or discussion."

Another finding indicates that vital treatments such as dental treatment, and rehabilitation responsibilities are allocated at various authorities on different governmental levels. A healthcare professional from a municipality in Jutland said:

"This [the dentists] we have nothing to do with. So, I believe that it is the responsibility of the hospital. We have nothing to do with that."

This impedes the already decentralized municipal rehabilitation offer even further due to the uncertainty of distribution of responsibilities.

DISCUSSION

The aim of this study was to elicit the needs of stakeholders during HNC rehabilitation in a Danish context. Our findings suggest that HNC patients and stakeholders have interrelated needs. Service delivery needs have been addressed by the patients and the stakeholders as the area that posed multiple challenges. A need for emotional support has been addressed by both the patients and informal caregivers, while social needs have been reported from the perspective of patients. The main physical challenge that has been found in this study concerns dysphagia.

Service Delivery Needs

Joint service delivery challenges have been addressed by HNC patients and stakeholders. These challenges concern unmet needs related to lack and organization of information. We found a relationship between the lack and organization of information provided for the patients in different phases of rehabilitation, and the paper format and volume of information. The provision of information in paper copies, has been seen as a factor that can lower the usability and the perceived value of information (47). Digital interactive interventions and tailor-made communication have been proposed as effective approaches for information provision to patients and informal caregivers (48–51). Digital information delivery could provide means to tackle information overload, for example, through personalization and contextualization of content. A recent multi-institutional study concluded that HNC patients prefer multiple modes of information delivery (72%), with one-to-one consultation being the most preferred method for cancer education followed by internet-based interventions (52).

Cancer patients have specific information requirements, requesting oftentimes to receive as much information as possible related to their cancer and its treatment (29, 53, 54). Information about diagnostic tests and treatment options have been reported as those areas in which patients and caregivers request to receive more information than they currently get. However, there is a lack of specialized learning resources and services to cover these ongoing needs (17, 33, 55). In line with this, our participants expressed a need for reception of supplementary or more comprehensive information in regards to swallowing exercises and dental treatments, confirming findings of other researchers (56). The provision of explanatory context and support from healthcare professionals, as well as information consistency have been valued as factors that could increase patients' satisfaction (57). The design of information systems for HNC patients is a highly complex process underpinned not only by the complexity of the disease itself, but also from the unique requirements and needs of each patient (31, 58–61).

Another endorsed problem concerns information management from the perspective of patients and informal caregivers. They argued that managing and having an overview of information has been challenging. Information management issues, such as the difficulty to keep track of all the treatments and interventions needed during rehabilitation can be attributed to the non-digital format of information and the lack of an efficient information management service. Journeys that cancer patients undergo are often fragmented and highly individual (62). Personalization of services and patients' involvement in the design process of interventions has been suggested as a way to identify changing needs within rehabilitation processes and to point out the specific moments that patients experience challenges (59, 63, 64). Effective communication between healthcare professionals and patients have been seen to have positive effects on patients' health, highlighting therefore that inclusion of patients in the design of services could be beneficial in different aspects (65).

Stakeholders participation in the design of rehabilitation services could also contribute towards more efficient re-allocation of organizational and structural responsibilities. In Denmark the responsibilities are allocated at various authorities on different governmental levels, with municipalities holding the

responsibility for the generalized rehabilitation interventions, while the hospitals for the specialized rehabilitation services for patients with cancer (24, 25). This organizational structure had been seen as a factor that can cause dissatisfaction, confusion and decreased the trust of patients, as it is not always transparent who holds each responsibility. The municipalities do not possess specialized proficiency needed to elaborate rehabilitation of HNC patients, due to discrepancies in the number of cancer incidences across the country (66). Knowledge and professional skills are divided across great distances, which often hinders closer collaboration across professional groups. Seamless collaboration between hospitals and municipalities could improve patients' experience and contribute towards establishing a trustful relationship between the two parties (66).

Besides organizational and structural problems, healthcare professionals who are working in municipalities located outside the Danish capital, point out an unmet need for knowledge sharing between healthcare professionals. They proposed that peer-to-peer knowledge sharing could elicit essential and updated treatment information. Knowledge generation practices through reflection on clinical experiences, or working relationships are sources of information that healthcare professionals can benefit from, nevertheless under-utilization of sharing professional experiences and communication gaps between healthcare professionals are common practices in healthcare (17, 67). Treatment decisions in HNC are critical and require the establishment of a multidisciplinary team of healthcare professionals (68). The challenges of providing consistent speech pathology care to regional/rural patients, as well as supporting clinicians with less experience in services or with less exposure to HNC utilizing telehealth is an approach used in other countries such as Australia (69). In addition, healthcare professionals argued that knowledge input from HNC patients about their rehabilitation experience and needs, could lead towards service improvements. Based on these findings Sundhed.dk and CKSK are planning to create an information portal for patients with HNC, which will facilitate patients, informal caregivers and healthcare professionals during HNC rehabilitation.

Emotional Needs

Emotional needs from the perspective of patients and informal caregivers include a desire for mutual psychological support. Emotional support is positively related to quality of life among cancer patients (70). Patients with deterioration in quality of life perceived a larger decrease in emotional support than patients with a positive course. HNC patients argued that emotional support enables them to continue practicing the swallowing and physical exercises. Therefore, motivation for rehabilitation and emotional support were found to have a positive relation. Similar to McEwen et al.'s findings, our HNC patients asserted also that a contact person motivated them to keep up with rehabilitation and supported them to coordinate all the interventions (17). Nevertheless, the emotional distress of newly diagnosed patients is related also to other factors such as the marital status and patients' lifestyle (8). Half of our participants stated that they were living alone, a fact that can have a negative impact on their emotional needs. As for informal caregivers, they felt that the burden of supporting their beloved ones had an impact on their wellbeing. Literature supports that caregivers often neglect their own health as they have the notion that the patient should be the center of attention (71), therefore an increasing attention should be given to comprehend the effects this has on the mental and psychological aspects of HNC caregiving. Due to the severity of side-effects such as the ability to speak (72), HNC caregiving poses a high risk of post-traumatic stress disorders and anxiety (17, 73–75). According to Howren et al. (76), interventions for HNC caregivers is still in its infancy, therefore future healthcare interventions should be focusing on accommodating their needs.

Social Needs

In line with previous studies, a need for social support has been also expressed by the informal caregivers and patients that participated in our study (32, 33). Informal caregivers can support and encourage patients that undergo post-treatment changes such as speech disorders, nutrition difficulties or facial features changes. Besides that, social inclusion of patients can contribute towards better functionality and better quality of life after treatment (77). As for patient-to-patient support, according to our findings, patients reported that the social support from peers had a positive impact on motivating them to continue performing physical exercises (78). HNC patients prefer to socialize with patients perceived as similar to them (30). The similarity of characteristics is based on individuals and on variables that patients perceive as relevant to their condition e.g. age, treatment, common experiences etc. (79). For example, breast cancer patients might identify themselves with everyday women, who cope with cancer rather than with the super-copers presented on television programs. These super-copers are usually famous women with cancer, who present that nothing has changed after cancer treatment (79). Moreover, the need to socialize with other patients similar to them is connected to minimization of feelings of deviance (80, 81). This also relates directly to the disease-free space the patients wish to have. Healthy people may ask more questions about a patient's condition or even start treating them differently than before the diagnosis. This behavior change is related to various reasons such as feelings that the other person is sick and therefore in need of help, difficulties to understand the condition or fear of feeling uncomfortable. The reaction of the social environment to the person with the diagnosed condition can be a reminder of the condition to the patients.

The social aspect during rehabilitation of HNC patients has been found to be influential also for informal caregivers, as they reported negative feelings such as guilt and shame. These feelings may be common to people close to a victimized person (i.e. a person who presided as a victim by a particular situation in this case cancer). Husbands of female breast cancer patients seem to deal with their negative feelings by perceiving themselves to be better than the norm, even if that was untrue (75, 78). According to Taylor et al. (82), only 4% of female breast cancer patients were abandoned by their husbands (82). However, the husband who is leaving his sick

wife was believed to be the norm by the informal caregivers (i.e. husbands). In general, comparing with people worse than one-self can lead to self-enhancement and better coping with the difficult situation (83). Psychosocial interventions have been concentrating on providing support to patients only for a limited time after the end of treatment. Nevertheless, in the cases of patients with HNC the development of long-term interventions is crucial (33, 84).

The need for social connections is in line with the Self-Determination Theory (85) arguing that 'relatedness' - the feeling of being understood, trusted and cared for by others, is one of the most important basic psychological needs for fostering wellbeing and enhancing motivation and sustainable behavior change.

Physical Needs

The main physical challenge that emerged in this study concerns eating difficulties, as a side-effect of medical treatment. This should be related to the fact that all four participants of our study received radiation therapy. Interventions that may improve the problems associated with nutrition after HNC treatments are essential and have direct effects on quality of patients' life (86). Current practice for managing dysphagia in HNC can include structured swallowing exercises usually given by a speech and language therapist prior and following cancer treatment (5, 21). In addition, mobile interventions, asynchronous telepractice applications for swallowing therapy like "SwallowIT" or screening tools for detection of swallowing, nutrition and distress status have been proposed as possible solutions to facilitate HNC patients' needs (87–89). Strength-based exercises and range of movement exercises (maneuvers) aimed at the swallowing musculature may prevent muscle atrophy and improve prognosis for oral intake (90–92). However, patient adherence to swallowing exercises is often poor (93–95). Devices supporting the exercises have proven efficient e.g. IQoro (96), however, improved adherence may be achieved by facilitating a change in patient behavior (5) and focusing on the psychological and/or social aspects of eating and drinking and not only on the functional aspect (92, 93). A recent systematic review to identify behavioral strategies in swallowing interventions, has found that behavior change techniques that occurred more frequently in effective interventions were; practical social support, behavioral practice, self-monitoring of behavior and credible source, such as a skilled clinician delivering the intervention (5).

Implications for Design and Future Work

The findings of this study highlight the different stakeholders' needs in the rehabilitation process, and pave the way for different and specific CH interventions that could address some of the HNC needs. For example, based on the findings, students identified several design opportunities in the form of questions: How do we ensure that patients have the opportunity to receive answers for spontaneous questions? How might we 'educate' relatives to behave more naturally and not pity or feel sorry for the patient? How might we support patients to overcome the social challenges related to eating in public? How can we better prepare patients for their individual treatment and the challenges that come with it? How might we help caregivers to feel less guilty and more supported? How can we create a service that encourages patients to perform their exercises outside the center? Students also proposed initial design ideas and concepts such as developing a "digital colleague" that provides professional knowledge for healthcare professionals about HNC and creating an online community for both healthcare professionals and patients, to share their experience on HNC rehabilitation. CH interventions have the potential to support the creation of holistic, personalized and inclusive solutions to tackle the diverse and complex needs of HNC patients (35).

Based on our findings, future research should explore possibilities of CH solutions to support the psychological wellbeing of both the informal caregivers (a safe place that enables them to share their thoughts, frustrations and guilt feelings) and for patients to gain strength from people in a similar situation. In addition, digital solutions to smoothen the care pathways would be a promising area of research. Especially, the transition from cancer care to rehabilitation could be eased with digital solutions that would guide the patient through the transition. The first steps of the rehabilitation process which are characterized by overload of information could benefit from solutions that would gradually personalize the rehabilitation information and actions by integrating them into the everyday lives of the patients. The current Covid-19 outbreak poses new challenges, such as the minimization of patient-staff contact time to reduce the risk of virus transmission (97). The pandemic outbreak is an opportunity to reconsider HNC cancer management, focusing on delivering CH solutions that will support the provision of rehabilitation support from a multidisciplinary team of healthcare professionals (98, 99). For example, CH solutions for swallowing-therapy exercises utilizing video communication and sensors during HNC rehabilitation is a successful intervention used in the postCovid-19 period (35). The value of such CH rehabilitation solutions for HNC is recognized during the pandemic, paving the way to novel care delivery methods (98).

We plan to continue our collaboration with the stakeholders and take forward some of the proposed concepts and ideas. In addition, we are considering a follow-up study with HNC in the UK to evaluate if some of the proposed solutions could be relevant or adjusted to the UK population.

Limitations

Our study has a number of limitations. The limited number of participants and the specific types of HNC and medical treatments might have introduced a bias in the findings. In addition, omitting information with regards to the patients' surgery and treatment type (e.g., type of surgery, site of tumor or radiation dose) may have resulted in exclusion of specific patients' rehabilitation needs. For example, a patient who underwent total laryngectomy could have different needs compared to a patient who underwent partial glossectomy. Similarly, including only patients who were capable of conducting lengthy and rigorous interviews may have excluded participants with severe communication difficulties, who may have additional needs that were not captured. However, rehabilitation needs are unique in nature and depend on a

variety of factors (e.g., age, severity, treatment type, family support, socio-economic state, individual characteristics, etc). Our aim was to elicit and identify the more holistic needs that might be common across different HNC patients. The qualitative nature of this research increases the credibility of the results, as it provides in-depth content focusing on the needs of a group. One can argue that our findings represent the primary needs of stakeholders during the rehabilitation of HNC patients. In addition, the secondary synthesis of data, the multiple different data collection processes and people engaged to collect the data might have introduced bias. Further research could observe later stages of the service design process, for example observing experiences of stakeholders after the implementation of a digital intervention supporting the rehabilitation process. Despite these limitations, the study is a baseline for future endeavors, as research addressing the interrelated needs of stakeholders during rehabilitation of HNC patients is still in its early stages.

CONCLUSIONS

While preliminary, this study offers insights related to the needs of patients and stakeholders during the HNC rehabilitation. Our findings point out that stakeholders' needs are interrelated. All the stakeholders faced service delivery challenges contextual to lack and organization of information, as well as to information sharing and management. The distribution of responsibilities between municipalities, hospitals and rehabilitation services raised additional challenges, suggesting that reallocation of responsibilities could alleviate this issue. Interrelated emotional and social needs have been found for HNC patients and informal caregivers, underlining the importance of inclusion of all actors in the design of future healthcare interventions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All the authors have been involved in analysis and interpretation of data. MK has been responsible for drafting the first version of the manuscript and coordinating writing. TP, VM, and MI have been involved in revising the manuscript critically for important intellectual content. Portions of this manuscript were presented at the 13th EAI International Conference on Pervasive Computing Technologies for Healthcare (100). All authors contributed to the article and approved the submitted version.

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Weekly Paclitaxel, Carboplatin, and Cetuximab as First-Line Treatment of Recurrent and/or Metastatic Head and Neck Squamous Cell Carcinoma for Patients Ineligible to Cisplatin-Based Chemotherapy: A Retrospective Monocentric Study in 60 Patients

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Objective: For most patients suffering from recurrent and/or metastatic head and neck squamous cell carcinoma (R/M HNSCC), chemotherapy is the main option after considering surgery and reirradiation. Cetuximab combined with a platinum-fluorouracil regimen (EXTREME) has been the standard of care for over a decade. Nevertheless, a significant number of patients remain unfit for this regimen because of age, severe comorbidities, or poor performance status. The aim of this study is to investigate an alternative regimen with sufficient efficacy and safety.

Methods: We reviewed retrospectively the medical charts of all patients treated with paclitaxel, carboplatin, and cetuximab (PCC) at our institution. Eligibility criteria were as follows: first-line R/M-HNSCC of the oral cavity, oropharynx, hypopharynx, or larynx not suitable for local therapy, cisplatin, and/or 5-FU ineligibility, ECOG-PS: 0–2. PCC consisted of paclitaxel 80 mg/m^2, carboplatin AUC 2, and cetuximab at an initial dose of 400 mg/m^2 then 250 mg/m^2, for 16 weekly administrations followed by cetuximab maintenance for patients for whom a disease control was obtained. The primary endpoint was overall survival (OS), and secondary endpoints were overall response rate (ORR), progression free survival (PFS), and safety.

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Results: We identified 60 consecutive patients treated with PCC between 2010 and 2016 at our institution. Thirty-one patients (52%) were ECOG-PS 2. Fifty-five patients (92%) were cisplatin ineligible. ORR was 43.3% (95% CI, 30.8–55.8), and disease control rate was 65% (95% CI, 52.9–77.1). With a median follow-up of 35.7 months (IQR 28.6–48.8), median PFS was 5.8 months (95% CI, 4.5–7.2), and median OS was 11.7 months (95% CI, 7.5-14.8). For ECOG-PS 0–1 patients, median OS was 14.8 months (95% CI, 12.2–21.7) while it was only 7.5 months (95%CI: 5.5-12.7) for ECOG-PS 2 patients ($p < 0.04$). Grades III–IV toxicities occurred in 30 patients (50%). Most toxicities were hematologic. Six patients (10%) had febrile neutropenia. Nonhematologic toxicities were reported such as cutaneous toxicities, neuropathy, infusion-related reactions, or electrolyte disorders.

Conclusion: The weekly PCC regimen seems to be an interesting option in cisplatin-unfit patients. This study shows favorable PFS and OS when compared with what is achieved with the EXTREME regimen and a high controlled disease rate with predictable and manageable toxicities even in the more fragile population.

Keywords: head and neck squamous cell carcinoma, recurrent or metastatic, chemotherapy, cetuximab, paclitaxel, carboplatin, first-line

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represent more than 90% of the head and neck tumors. In Europe, approximately 140,000 new cases were diagnosed in 2014, corresponding to an annual incidence of 43/100,000. The main risk factors of HNSSC are tobacco with alcohol heavy/frequent consumption and HPV infection (1). In France, most patients are active or former smokers frequently in association with a high consumption of alcohol. Thus, they are likely to suffer from several active tobacco/alcohol-related comorbidities, undernourishment, and other active carcinomas. Considering significant concomitant nonmalignant diseases, age and general condition are crucial in oncological decision-making because a vast majority of patients turns out to be ineligible for clinical trial or standard of care (2).

Concerning recurrent and/or metastatic squamous cell carcinoma of head and neck (R/M HNSCC), palliative chemotherapy is the standard of care if local treatment (surgery or radiotherapy) cannot be curative. There is a need to find an optimal strategy to achieve the highest possible overall survival and patient's quality of life. In 2008, the EXTREME trial (3) showed the benefits of adding cetuximab to a platinum-5-FU chemotherapy in R/M HNSCC first-line treatment. An overall response rate (ORR) of 36% was achieved, median progression free survival (PFS) was 5.6 months, and median overall survival (OS) was 10.1 months. The EXTREME regimen has emerged as the standard of care for fit R/M HNSCC patients. Nevertheless, numerous patients remain unfit for this regimen because of frailties such as age, ECOG-PS >1, or heavy comorbidities, as evidenced by 82% of grades III–IV toxicities (3).

Other treatments consisting of a platinum-based chemotherapy associated with taxanes (docetaxel or paclitaxel) were investigated for HNSCC including R/M HNSCC (4). In a phase II trial (5), docetaxel combined with a cisplatin and cetuximab regimen (TPEx) achieved promising outcomes with a 44.4% ORR and a 79.6% disease control rate (DCR). Thus, the same authors carried out a phase II randomized trial (TPExtreme), comparing TPEx with EXTREME in terms of efficacy and safety (6). Results suggest that taxanes are an option in first-line treatment. However, this regimen should be used exclusively in cisplatin fit patients.

Therefore, some studies investigated alternative polychemotherapies in nonfit patients. Carboplatin with weekly paclitaxel is a safe and recommended option in the elderly population affected by advanced nonsmall cell lung cancer (7). In R/M HNSCC as well, some smaller nonrandomized studies demonstrated that first-line weekly carboplatin and paclitaxel could be safely used and improved efficacy when compared with monotherapy schedules in unfit patients (8). The paclitaxel, carboplatin, and cetuximab (PCE) regimen showed a 40% ORR, 5.2 months median PFS, and a 14.7-month median OS as first-line treatment in R/M HNSCC patients (9). The weekly paclitaxel, carboplatin, and cetuximab (PCC) regimen was first reported with promising results by Kies et al. in the locally advanced setting with a high dose of paclitaxel (10). The aim of this study is to provide a deeper insight into the weekly PCC efficacy and safety in the first-line R/M setting.

PATIENTS AND METHODS

Patient Selection

We retrospectively reviewed data from medical charts at our institution (Centre Paul Strauss, Strasbourg, France) between January 2010 and December 2016 to identify patients treated with paclitaxel, carboplatin, and cetuximab and suffering from histologically confirmed SCC of the oral cavity, oropharynx, larynx, hypopharynx, or cervical lymph node from assumed

HNSCC. Patients with skin SCC and sinus or nasopharynx carcinoma with poor differentiation were not selected.

Adult patients (aged 18 or older) in first-line treatment of a metastatic and/or recurrent HNSCC with no curative intent, ECOG-PS 0-2, and cisplatin and/or 5-FU ineligibility were included. Patients were considered cisplatin ineligible if at least one of the following criteria was met: age ≥70 or ECOG-PS 2 or creatinine clearance <60 ml/min or significant active comorbidities or cisplatin free interval <6 months. Patients were considered 5-FU ineligible in case of severe cardiovascular previous history including coronary insufficiency whether or not complicated by myocardial infarction, heart insufficiency, or lower limb arteriopathy of at least stage II. A treatment by radiotherapy or surgery for a previous locoregional relapse was permitted.

Induction regimen and palliative second-line or further treatment by PCC were excluded of the analysis.

The selection process is shown in **Figure 1**.

Treatment

From January 2010 to January 2012, we administered the PCC regimen as follows: carboplatin AUC 5 on day 1; paclitaxel 80 mg/m² on days 1, 8, and 15; and cetuximab 400 mg/m² loading dose then 250 mg/m² weekly. This pattern was repeated on day 22. A maximum of six cycles was administrated followed by a cetuximab maintenance given weekly or biweekly until progressive disease or unacceptable toxicities. However, administering carboplatin once every 3 weeks caused hematologic toxicity to such an extent that it became frequently impossible to administer paclitaxel on D15 and even sometime on D8.

Thus, from February 2012 to December 2016, the carboplatin infusion schedule was modified switching to a weekly administration: weekly carboplatin AUC 2 (maximum dose of 220 mg); paclitaxel 80 mg/m²; and cetuximab 400 mg/m² loading dose then 250 mg/m². A maximum of 16 cycles was performed followed by a cetuximab maintenance given weekly (250 mg/m²) or every 2 weeks (500 mg/m²) until progressive disease or unacceptable toxicities.

Doses could be reduced initially or during treatment according to patient's comorbidities or toxicities.

Assessment

The efficacy of the protocol was assessed on the basis of response rate, progression-free survival and overall survival. A computed tomographic scan (or 18F-FDG PET/CT if needed) was performed at baseline, then every eight weeks. Measurements were compared between baseline and 8th-week CT scan (or 18F-FDG PET/CT) according to RECIST 1.1 criteria. The ORR is the complete response (CR) rate plus the partial response (PR) rate. The DCR is the ORR plus the stable disease (SD) rate. Radiologically, unevaluable patients were considered progressive if clinical reports mentioned it.

Toxicities were monitored weekly throughout the treatment and evaluated using the Common Terminology Criteria for Adverse Events version 4.0. Dose intensity data were calculated in order to assess regimen feasibility.

Statistical Analysis

PFS (time from first PCC infusion to progression or death) and OS (time from first PCC infusion to death) were estimated by the Kaplan-Meier method. If progression or death did not occur before the cutoff date, data were censored at the time of the last valid assessment.

The follow-up time is calculated from first PCC infusion to data cutoff (16 Jun 2018).

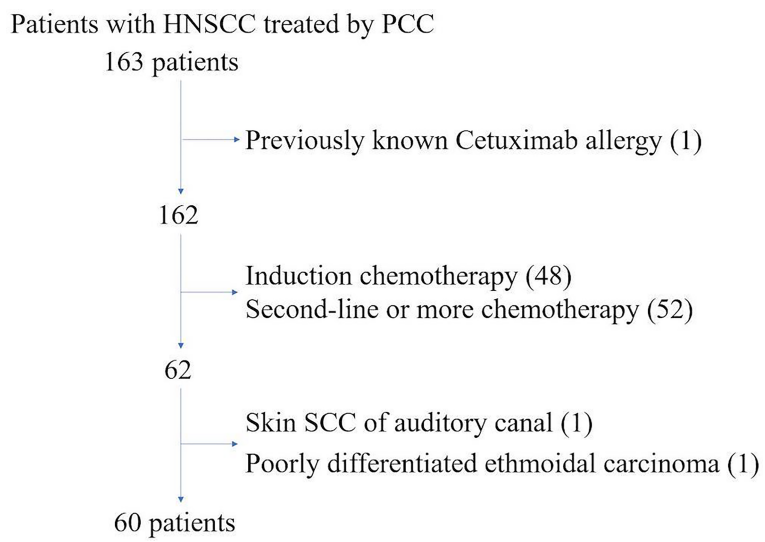

FIGURE 1 | Selection process.

RESULTS

Patient Characteristics

Sixty patients were treated with the first-line combination of paclitaxel and carboplatin plus cetuximab for a R/M HNSCC at our institution between January 2010 and December 2016. Median age was 61, with 10 patients (17%) aged 70 or more. Sex ratio was 5:1. In our study, the main risk factor was tobacco smoking as 80% of patients were former or current smokers. HPV infection was only assessed in two patients with oropharyngeal SCC by using p16 immunohistochemistry as a surrogate marker: one patient was p16 positive, the other one was not.

Five patients were diagnosed with distant metastases at the initial assessment and received PCC as a first treatment. Fifty-five patients had been pretreated with surgery and/or chemoradiotherapy. Fifteen patients in a recurrent setting had received locoregional treatments with a curative intent (such as surgery or reirradiation).

Forty-six patients (77%) were diagnosed with a locoregional relapse, among whom 38 patients in the field of an earlier irradiation. Twenty-nine patients (48%) had been already treated with platinum-based regimen in a neoadjuvant setting (18%) and/or with concurrent radiotherapy (32%) as a multimodality treatment of their initial tumor. Platinum-free interval was less than 3 months in 11 patients (18% of the whole patient population), between 3 and 6 months in three patients (5%) and longer than 6 months in 15 patients (25%). Because of a cisplatin-related kidney failure after a single course of TPEx, one patient received subsequently a PCC regimen. Patient characteristics are summarized in **Table 1**.

Twenty-nine patients (48%) were ECOG-PS 0–1 and 31 patients (52%) were ECOG-PS 2 at treatment onset. Frailty characteristics such as undernourishment and active comorbidities are reported in **Table 2**.

As defined in the inclusion criteria, 55 patients (92%) were ineligible to cisplatin. Thirty-four patients (57%) were ineligible to 5-FU because of severe cardiovascular comorbidities.

A second primary cancer arose in six patients during follow-up: two patients with a nonsmall cell lung cancer, one patient with a cutaneous melanoma and a nonsmall cell lung cancer, one patient with a hepatocellular carcinoma, one patient with a cutaneous squamous cell carcinoma, and one patient with a prostate adenocarcinoma.

PCC Delivery

Among the 60 patients included, six were treated with the first pattern to be used (carboplatin AUC 5 every 3 weeks, weekly paclitaxel 80 mg/m², and cetuximab 400 mg/m^2 initial dose, followed by weekly 250 mg/m^2). Starting in 2012, the 54 following patients were treated with weekly carboplatin AUC 2 (maximum dose of 220 mg: 49 patients were involved in dose limiting), paclitaxel 80 mg/m², and cetuximab 400 mg/m² loading dose then 250 mg/m². A maximum of 16 cycles was performed followed by a maintenance administration of cetuximab given weekly (250 mg/m²) or biweekly (500 mg/m²) until progressive disease or unacceptable toxicities.

A first clinical and radiological evaluation was done after eight cycles of PCC. As shown in **Table 3**, seven patients (12%) did not resume chemotherapy due to unacceptable toxicities and 16 patients (27%) because of progressive disease. A focal treatment (neck and/or metastasis) was carried out in seven patients (12%) because of a particularly good partial response. Cetuximab maintenance began after this assessment in eight patients (13%).

Median number of delivered cycles was 9.5 for chemotherapy and 10.5 for cetuximab. Twenty-four patients (40%) completed the 16 cycles of treatment, of whom 17 patients (28%) with the three drugs, while carboplatin or paclitaxel had to be stopped in seven patients.

Doses of paclitaxel and/or carboplatin and/or cetuximab had to be reduced in 19 patients, 37 patients, and three patients, respectively. Forty-five percent of patients experienced delayed chemotherapy due to side effects. PCC had to be stopped in 16 patients (27%) because of severe toxicities.

Toxicities are reported in **Table 4**. Thirty patients (50%) showed grades III–IV toxicities. Most toxicities were hematologic. Blood transfusions were required in 18 patients (30%). EPO and G-CSF were used as secondary prophylaxis in respectively nine (15%) and 30 patients (50%). Sixteen unexpected hospitalizations occurred due to infection, including six febrile neutropenia (10%). Four infections (6.6%), mostly pneumopathies, led to death which occurred only in ECOG-PS 2 patients. No other toxicity brought toxic death about. We observed 15 grades III–IV (25%) nonhematologic toxicities. Hypokalemia and hypomagnesemia are the most noticeable nonhematologic toxic effects in our study.

PCC Efficacy

PCC achieved a 43.3% (i.e., 26 responses) ORR (95% CI: 30.8–55.8) with three complete responses and 23 partial responses. Thirteen patients experienced stable disease. DCR came out at 65% (i.e., 39 controlled patients) (95% CI: 52.9–77.1). Progression occurred in 16 patients (26.7%): seven patients experienced clinical progression but could not be radiologically evaluated, six patients experienced CT-scan-proved disease progression, and three patients showed dissociated responses with appearance of new metastases despite partial responses on target lesions. Five patients were not evaluable because of nonmeasurable lesions (**Table 5**). The ORR is similar in the 38 patients with a locoregional relapse in a previously irradiated area; in this population, we observe 14 responses, i.e., a response rate of 36.8%. Among the 14 patients for whom the cisplatin free interval was less than 6 months, we observed three partial responses.

Change in target lesions was not evaluable in 12 patients: five patients died before evaluation (four due to progressive disease, one due to infection); one patient was not compliant; one patient could not be re-evaluated due to clinical deterioration; and five patients did not have any measurable lesion according to RECIST criteria. Change in target lesions is shown in **Figure 2**.

With a median follow-up of 35.7 months (IQR 28.6–48.8), we observed a median OS of 11.7 months (95% CI: 7.5–14.8) and a median PFS of 5.8 months (95% CI: 4.5–7.2). Kaplan-Meier curve-line estimate of PFS and OS are shown in **Figures 3**, **4**.

TABLE 1 | Baseline characteristics of the patients.

Variable		*N* = 60
Gender [*n* (%)]	*n*	60
	Male	50 (83)
	Female	10 (17)
Age (years)	Median	61
	Range	23–79
Age [*n* (%)]	*n*	60
	<65 years	36 (60)
	65–69 years	14 (23)
	≥70 years	10 (17)
ECOG-PS [*n* (%)]	*n*	60
	0	9 (15)
	1	20 (33)
	2	31 (52)
Tobacco status [*n* (%)]	*n*	60
	Nonsmoker	12 (20)
	Current or former smoker	48 (80)
Primary tumor localization [*n* (%)]	*n*	60
	Oropharynx	23 (40)
	Oral cavity	17 (28)
	Hypopharynx	12 (20)
	Larynx	7 (12)
	Unknown	1 (2)
Histologic type [*n* (%)]	*n* (not specified or missing)	38 (22)
	Well differentiated	8 (21)
	Moderately differentiated	23 (60)
	Poorly differentiated	7 (18)
Initial treatment [*n* (%)]	*n*	60
	Neoadjuvant chemotherapy + Surgery	5 (8)
	Neoadjuvant chemotherapy + Surgery + CRT	1 (2)
	Neoadjuvant chemotherapy + CRT (cetuximab)	5 (8)
	Surgery	9 (15)
	Surgery + RT	6 (10)
	Surgery + CRT (platin-based)	15 (25)
	Surgery + CRT (other)	7 (12)
	RT alone	2 (3)
	CRT (cisplatin)	3 (5)
	CRT (cetuximab)	2 (3)
	No prior treatment	5 (8)
Local treatment for first relapse with a curative intent [*n* (%)]	Surgery	11 (18)
	Reirradiation	4 (7)
Tumor extension at baseline [*n* (%)]	*n*	60
	Loco regional only	33 (55)
	Loco regional and metastatic	13 (22)
	Metastatic only	14 (23)
Characteristics of relapse [*n* (%)]	*n*	60
	Relapse in RT field	38 (63)
	Relapse after platinum-based regimen (neoadjuvant, CRT)	29 (48)
Platinum free interval before baseline [*n* (%)]	*n*	29
	<3 months	11 (38)
	3–5.9 months	3 (10)
	≥6 months	15 (52)
Chemotherapy ineligibility [*n* (%)]	*n*	60
	Cisplatin	55 (92)
	5-FU	34 (57)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; CRT, concurrent chemoradiotherapy; RT, radiotherapy.

In the ECOG-PS 0–1 population (i.e., 29 patients), median OS was 14.8 months (95% CI: 12.2–21.7) and median PFS was 7.1 months (95% CI: 6.3–9.0 months). In ECOG-PS 2 patients (i.e., 31 patients), median OS was 7.5 months (95% CI, 5.5–12.7; $p < 0.04$) and median PFS was 4.6 months (95% CI: 3.5–6.9; $p = 0.07$).

DISCUSSION

Our study shows that in first-line R/M HNSCC, a combination of paclitaxel, carboplatin, and cetuximab makes it possible to achieve results comparing favorably with what may be obtained through chemotherapies based on platinum-5-FU and

TABLE 2 | Frailty criteria of patients.

List of frailty criteria [*n* (%)]	*n*	60
	Age >70 years	10 (17)
	ECOG-PS = 2	31 (52)
	Undernourishment[a]	45 (75)
	Significant active associated comorbidities	
	Severe atheroma	32 (53)
	Heart insufficiency	10 (17)
	Chronic obstructive lung disease, ≥ stage 2	19 (32)
	Kidney insufficiency	2 (3)
	Pre-existing neuropathy	5 (8)
	Previously cured cancer	17 (28)
	Synchronous active cancer	6 (10)
	Others (psychiatric disorder, cirrhosis, organ transplant, etc.)	35 (58)
Number of criteria [*n* (%)]	*n*	60
	None	1 (2)
	1 criterion	10 (17)
	2 criteria	12 (20)
	3 or more criteria	37 (62)

[a]*Undernourishment: albumin <30 g/L or weight loss over 5% in 6 months or weight loss over 2% if BMI >20 or BMI <18.5 or BMI <21 in 70 years and more aged patients.*

TABLE 3 | PCC delivery before cetuximab maintenance.

Variable	*N* = 60	Paclitaxel 80 mg/m²/week	Carboplatin AUC2/week	Cetuximab 400 mg/m² then 250 mg/m²/week
Number of cycles	Median	9.5	9.5	10.5
	Range	1–19	1–21	1–21
Early discontinuation of treatment (≤8 cycles):			24 (40)	
- Due to unacceptable toxicities	*n* (%)		7 (12)	
- Due to progressive disease	*n* (%)		16 (27)	
- Change of treatment (local treatment, etc.)	*n* (%)		7 (12)	
Delivery completed (≥16 cycles)	*n* (%)		24 (40)	
Patients with dose reductions	*n* (%)	19 (32)	37 (62)	3 (5)
Patients with ≥1 dose held for ≥7 days	*n* (%)	27 (45)	27 (45)	23 (38)
Dose intensity[a]	Median	65	1.6	250
	Range	40–80	0.8–2	125–250

AUC, area under the curve. [a]*Dose delivered per week, accounting for treatment delays and dose reductions. Units of measure are as follows: paclitaxel: mg/m²/week; carboplatin: AUC/week; cetuximab: mg/m²/week. The loading dose of cetuximab (i.e., 400*[a]*mg/m²) was not included in the calculation of dose density.*

TABLE 4 | Maximal toxicity per patient.

	Grade I-II	Grade III	Grade IV
Overall toxicities [*n* (%)]	21 (35)	30 (50)	
Non hematologic toxicities [*n* (%)]	14 (23)	15 (25)	
Cutaneous	12 (20)	7 (12)	0
Neuropathy	3 (5)	2 (3)	0
Electrolytes disorders	3 (5)	5 (8)	2 (3)
Infusion reaction	3(5)	1 (2)	0
Nausea	5 (8)	0	0
Diarrhea	4 (7)	1 (2)	0
Hematologic toxicities [*n* (%)]	17 (28)	21 (35)	
Neutropenia	30 (50)	8 (13)	7 (12)
Anemia	26 (43)	7 (12)	0
Thrombopenia	7 (12)	1 (2)	0
Toxicity-related data [*n* (%)]			
Blood transfusion		18 (30)	
EPO (secondary prophylaxis)		9 (15)	
G-CSF (primary prophylaxis)		4 (7)	
G-CSF required (secondary prophylaxis)		30 (50)	
Febrile neutropenia		6 (10)	
Hospitalisation due to infection		16 (27)	
Hospitalisation		26 (43)	
Deaths in association with AEs		4 (6,6)	

TABLE 5 | Efficacy after 8 weeks of treatment.

	n = 60
Overall response rate (95% CI)	43.3% (30.8–55.8)
Complete response	3 (5%)
Partial response	23 (38.3%)
Disease control rate (95% CI)	65% (52.9–77.1)
Stable disease	13 (21.7%)
Progressive disease	16 (26.7%)
Non evaluable	5 (8.3%)

cetuximab (3) or cisplatin-docetaxel and cetuximab (5, 6), and this, with particularly frail patients.

Indeed, in our study, 55 patients (92%) are cisplatin ineligible: ECOG-PS 2: 52%, platinum free interval <6 months: 23%, at least three frailty criteria: 62%, age ≥70: 17%; however, a 43.3% ORR, a 5.8-month median PFS, and a 11.7-month median OS are achieved.

Although a 11.7-month median OS compares favorably with the 10.1 months obtained through the EXTREME regimen (3), it seems however shorter than the 14.5 months observed with the TPEx (6) and the 14.7 months with the PCE regimens (9). It should be noted however that these two latter studies concern a more favorable population of patients ECOG-PS 0 or 1 and that patients who were enrolled into the TPExtreme study were cisplatin fit and under age 70. In our study, when we consider the ECOG-PS 0–1 patients, the 14.8 months median OS is very similar to that reported with the TPEx or PCE regimens.

Pêtre et al. reported on a weekly paclitaxel and carboplatin combination in a particularly frail and heavily pretreated population that produced a 40% ORR, a median PFS of 4.7 months, and a median OS of 9.1 months (11). Interestingly, this study confirms the major impact of cisplatin eligibility and ECOG-PS on survival outcomes: median OS is 13.7 months for cisplatin-eligible patients whereas it is only 8 months for cisplatin-ineligible patients. For cisplatin-ineligible patients, median overall survival decreases from 11.5 to 3.6 months in patients ECOG-PS 0–1 and ECOG-PS 2–3, respectively (11).

The number of administered weekly PCC cycles, cetuximab maintenance, and subsequent treatments seem to be important factors of survival. Indeed, in the retrospective study reported by Narveson et al, where treatment is limited to six weekly cycles of PCC, although a similar ORR of 37% is reported, median PFS is 4.6 months and median OS is only 5.25 months (12).

Weekly paclitaxel is a well-established regimen which allows high dose intensity with low hematologic toxicity (13). Likewise, fractionated administration of carboplatin allows also to decrease the hematologic toxicity and thus to maintain continuous weekly administration of chemotherapy with as few toxicity-related interruptions as possible (9, 11). Nevertheless, in our study, although toxicity is noteworthy, it is however mostly hematologic and may be managed. It is caused to a large extent by the frailty of the treated population. We observed 10% of febrile neutropenia, 50% of secondary prophylaxis using G-CSF, and 27% of hospital readmission for sepsis which resulted in four deaths (6.6%). It should be noted however that deaths in association with adverse events are only observed in ECOG-PS 2 patients. Likewise, in the TPExtreme study, a 7.7% rate of deaths in association with adverse events is reported in the EXTREME arm and 5.9% in the TPEx arm (6). We are now proposing G-CSF as a primary prophylaxis which significantly reduces infectious toxicity. Indeed, weekly administration of G-CSF is safe and effective as reported by Kies et al. in the first publication of the weekly PCC (10).

Results of our study like these observed with the TPEx and the PCE regimens as well as in the CETMET trial show that it is possible to replace advantageously 5-FU by a taxane. The CETMET trial is a randomized phase II study which shows that the replacement of 5-FU by paclitaxel allows to decrease toxicity: 60% of the grades III–V reported toxicities being in the EXTREME arm (p = 0.034). Moreover, authors observed an increasing trend in the median PFS from 4.37 months in the EXTREME arm to 6.5 months in the paclitaxel, carboplatin, and cetuximab arm (p = 0.064) (14). The randomized phase II study TPExtreme did not show however any survival advantage when

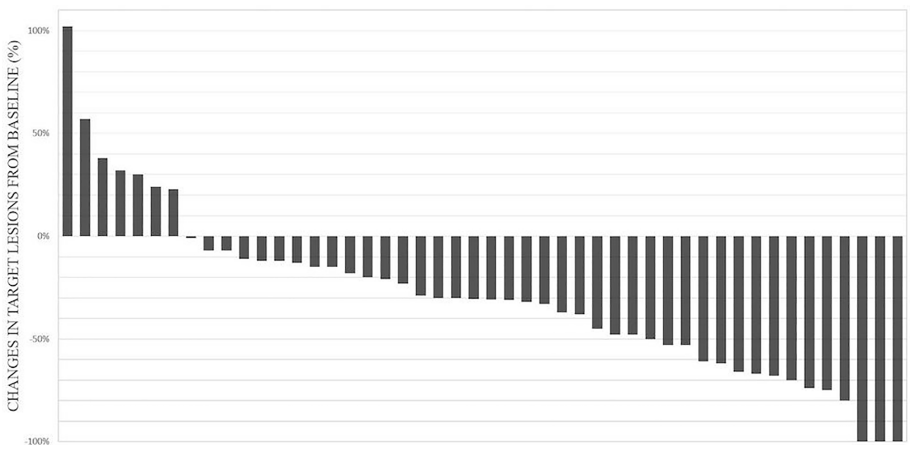

FIGURE 2 | Waterfall plot of 48 assessable patients for change in target lesions.

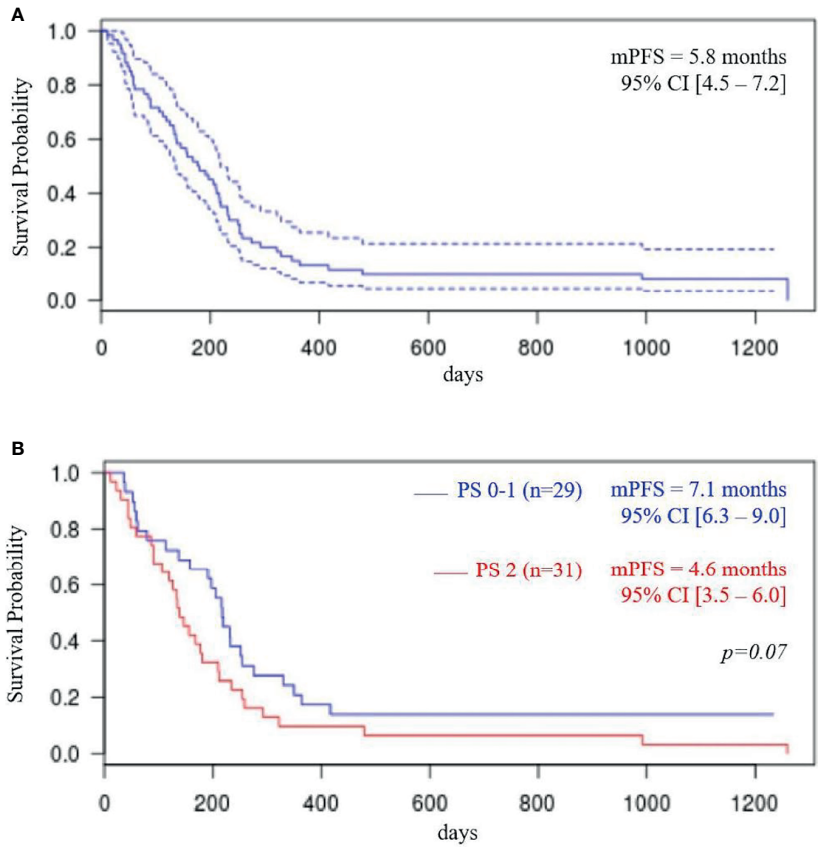

FIGURE 3 | Progression-free survival (PFS) total population **(A)**; PFS according to performance status (PS) **(B)**.

compared with the EXTREME protocol but confirms a high median survival of 14.5 months and a favorable safety profile in the TPEx arm (6).

The EXTREME protocol has remained the standard of care for first-line R/M until 2019 when the KEYNOTE-048 study has demonstrated, as far as survival is concerned, the superiority of immunotherapy by the anti-PD-1 pembrolizumab for the CPS ≥1 population and of the combination of pembrolizumab, platinum, and 5-FU in all patients (15). Nevertheless, platinum and cetuximab associations may remain relevant as first- or second-line R/M treatments in situations such as described hereunder. As first-line treatment, the association of platinum and cetuximab is appropriate when using an anti-PD-1 which is not suitable because of insufficient efficacy whenever the combined positive score (CPS) is inferior to 1 (which in Europe precludes its use in association with chemotherapy as well) or in case the patient is considered ineligible for immunotherapy particularly with an active autoimmune disease treated by immunosuppressive agents. In first-line treatment, there remains a need to address the problem of fragile patient, particularly cisplatin unfit or ECOG-PS 2 patients for whom the toxicity of the pembrolizumab combined to platinum/5-FU is too severe to be considered (85% of grades III–IV). Pembrolizumab alone may be proposed (55% of grades III–IV toxicity, 17% related to treatment), but the ORR is only 19% for the CPS ≥1 population (15) which appears inappropriate for severely symptomatic patients (16), whereas the response rate provided by the weekly PCC regimen is 43%. Moreover, the efficacy of pembrolizumab for ECOG-PS 2 patients has not been formally studied. As a second-line R/M treatment, following the administration of pembrolizumab alone, platinum and cetuximab combinations remain perfectly relevant and this especially since increased efficacy of chemotherapy following the use of anti-PD-1 agents has been reported (17). The weekly PCC may be then an interesting option for cisplatin-unfit patient, even for those who are ECOG-PS 2.

Despite its two main advantages (response duration and low toxicity), immunotherapy by pembrolizumab alone benefits only a minority of patients, and the determination of PD-L1 by combined positive score (CPS) remains an imperfect predictive factor (23% of response for CPS >20). Moreover, progression rate at first evaluation is high (around 40%) which makes it risky to propose immunotherapy alone to severely symptomatic patients. Combining pembrolizumab to chemotherapy makes it possible to improve results (ORR 36%, median OS of 13 months). There remain however two drawbacks: high toxicity (85% grades III–IV) and when compared with EXTREME, a survival benefit which is not clearly demonstrated for all subgroups (CPS <20) (18).

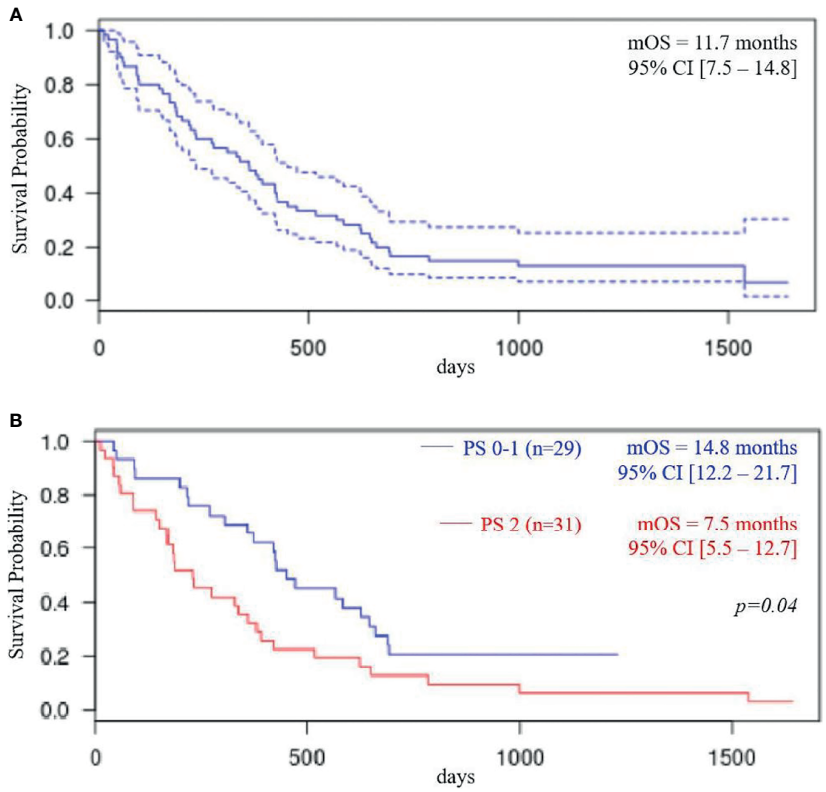

FIGURE 4 | Overall survival (OS) total population **(A)**; OS according to performance status (PS) **(B)**.

Considering the above, it remains clearly necessary to improve the immunotherapy combination or the associated chemotherapy. The combination of weekly paclitaxel, carboplatin, and durvalumab which is intended specifically for frail patients in first-line R/M is presently being studied in the frail-immune trial (19).

Combining monalizumab with cetuximab in at least second-line R/M patients pretreated with platinum, 45% of whom had also received an antiPD-1, has shown promising results which still remain to be confirmed in a randomized phase II study (20). A probable synergy of an anti-PD-1 with cetuximab (21) would justify the next step of studying a combination of PCC with an anti-PD-1 or also PCC with monalizumab.

In addition, the PCC regimen showed promising results in the neoadjuvant setting with an ORR ranging from 70% to 97% (10, 22, 23). Haddad et al. showed in a phase II randomized study, in the neoadjuvant setting, that weekly PCC is as effective and less toxic than cetuximab–Taxotere/platin/5-FU (C-TPF) making weekly PCC an option of choice for TPF-unfit patients (24).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HC and CB conceptualized and drafted the study and the manuscript. MB and AJ critically reviewed the manuscript. HC, MB, RF, CF, and MK collected patients' data. CB and PW reviewed all CT scans. AT realized statistical analysis. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: CB is advisory board consultant for BMS and Astra Zeneca, and has received honoraria for consulting from Merck, BMS, Astra Zeneca and MSD. MB has received honoraria for consulting from BMS, Ipsen and MSD.

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TERT Promoter Mutations and rs2853669 Polymorphism: Useful Markers for Clinical Outcome Stratification of Patients With Oral Cavity Squamous Cell Carcinoma

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Objective: To date, no useful prognostic biomarker exists for patients with oral squamous cell carcinoma (OCSCC), a tumour with uncertain biological behaviour and subsequent unpredictable clinical course. We aim to investigate the prognostic significance of two recurrent somatic mutations (-124 C>T and -146 C>T) within the promoter of telomerase reverse transcriptase (*TERT*) gene and the impact of TERT single nucleotide polymorphism (SNP) rs2853669 in patients surgically treated for OCSCC.

Methods: The genetic frequencies of rs2853669, -124 C>T and -146 C>T as well as the telomere length were investigated in 144 tumours and 57 normal adjacent mucosal (AM) specimens from OCSCC patients.

Results: Forty-five tumours harboured *TERT* promoter mutations (31.3%), with -124 C>T and -146 C>T accounting for 64.4% and 35.6% of the alterations respectively. Patients with -124 C>T *TERT* promoter mutated tumours had the shortest telomeres in the AM (p=0.016) and showed higher risk of local recurrence (hazard ratio [HR]:2.75, p=0.0143), death (HR:2.71, p=0.0079) and disease progression (HR:2.71, p=0.0024) with the effect being potentiated by the co-occurrence of T/T genotype of rs2853669.

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Conclusion: -124 C>T *TERT* promoter mutation as well as the T/T genotype of the rs2853669 SNP are attractive independent prognostic biomarkers in patients surgically treated for OCSCC, with the coexistence of these genetic variants showing a synergistic impact on the aggressiveness of the disease.

Keywords: oral cavity squamous cell carcinoma (OCSCC), telomerase, *TERT* promoter mutations, SNP rs2853669, telomere, prognostic biomarkers, survival

INTRODUCTION

With a worldwide estimated age-standardized incidence rate of 4.0 per 100,000 and an estimated number of new cases in 2018 of 354,864, oral cavity squamous cell carcinoma (OCSCC) is the most common carcinoma developing from the epithelial lining of the upper aero-digestive tract (UADT), thus representing an important burden on health care (1).

Based on the histopathological stage, OCSCC can exhibit an unpredictable behaviour with a fraction of patients with early-stage cancer suffering from poor prognosis (2). Patients curatively treated for OCSCC have indeed a high propensity to develop both recurrences and second field tumours (3). Thus, despite recent improvements in the management strategies of OCSCC, improvements in outcomes have been modest (4).

High-risk human papillomaviruses (HPVs), responsible for more than 50% of oropharyngeal squamous cell carcinomas (SCCs) and robust prognostic biomarkers in risk-stratifying in individuals with these malignancies (5), play only a marginal role in OCSCC (6). Thus, since not all OCSCCs are attributable to tobacco and alcohol exposure, the aetiopathogenesis of these neoplasms remains unknown in several cases and no reliable biomarker capable of stratifying the prognosis of OCSCC exists. It is, therefore, of paramount importance to identify biomarkers and molecular signatures predicting cancer relapse that may guide surveillance follow-up strategies and adjuvant treatments.

The infinite proliferation of malignant cells is a hallmark of oncogenesis and telomere/telomerase interplay dictates cell replicative capacity. Telomerase is indeed usually repressed in normal somatic cells, but it is detectable in the vast majority of tumours (7, 8). By synthesizing the telomere sequences and thus preventing cell senescence and apoptosis, the inappropriate activation of the catalytic component of the telomerase, telomerase reverse transcriptase (TERT), appears crucial for maintaining cellular replicative capacity and allowing tumour formation (9). Furthermore, through its non-canonical extra-telomeric functions, the re-activation of telomerase in cancer cells may affect cancer progression and metastasis (10, 11). These properties make TERT a potentially attractive biomarker in cancer.

Among the different mechanisms leading to the inappropriate reactivation of TERT in cancer, mutually exclusive recurrent C-to-T transitions at nucleotides 1,295,228 (-124 C>T) and 1,295,250 (-146 C>T) within the core promoter of *TERT* creating *de novo* binding sites for E-twenty-six (ETS) transcription factors and leading to increased *TERT* gene expression are particularly interesting: first, their prognostic role was consistently observed in several cancers (12), second, among SCCs of the UADT, *TERT* promoter mutations were observed to be topographically restricted to OCSCC (13), and third, unlike assessing *TERT* mRNA levels, *TERT* promoter mutations can be more easily analysed in formalin-fixed paraffin-embedded (FFPE) specimens from routinely collected biopsies.

Although a previous investigation conducted in a population of subject with OCSCC from Taiwan found that those harbouring the -124 C>T *TERT* promoter mutation had a worse prognosis, this was not statistically significant. However, current evidence suggests that the effect of *TERT* promoter mutations may be affected by a common single nucleotide polymorphism (SNP), rs2853669, within the *TERT* core promoter close to the hotspot mutation sites (14). The minor C-variant allele of the SNP disrupts a pre-existing ETS2 binding site at -245 bp in the *TERT* promoter region resulting in decreased TERT expression (15) and thus, counteracts the transactivation activity of the *TERT* promoter hotspots (14). A meta-analysis reports that among cancer patients with *TERT* promoter mutations, the rs2853669 T/T genotype confers a worse prognosis (16), but the modifying role of this SNP in the prognostic value of *TERT* promoter mutations is still controversial (12, 17–20). To date, the prognostic value of rs2853669 in OCSCC remains to be elucidated.

Thus, the main aims of this study were to investigate the prevalence and the clinical significance of *TERT* promoter mutations and the impact of the *TERT* rs2853669 SNP in a larger series of patients surgically treated for OCSCC.

MATERIALS AND METHODS

Patients and Tissue Samples

This is a multi-centre retrospective observational study conducted with the approval of the ethics committee of Treviso/Belluno provinces (Ethic vote: 346/AULSS9) and was performed in a cohort of 144 consecutive patients diagnosed with OCSCC from February 1, 2010 to September 30, 2018, who underwent up-front surgery with/without adjuvant (chemo) radiotherapy, whose samples were available for analysis. All patients gave their informed consent. The study network included three University Hospitals in Northeast Italy, located in Padova, Treviso, and Trieste.

Patients were routinely followed-up [median follow-up time: 43 months; interquartile range (IQR), 28-75 months] according to consensus guidelines with endoscopic examination of the

upper aero-digestive tract every 1–3 months for the first year, 3–4 months during the second year, 4–6 months during the 3rd year, and every 6 months thereafter. A dedicated CT scan of the chest was performed annually. Additional dedicated head and neck imaging was arranged based on clinical features and local protocol.

Data for 27 OCSCC samples (tumour tissue, adjacent mucosa and patient characteristics) were available from our previous study (13). One hundred and seventeen specimens were FFPE. Estimations of tumour cell content on FFPE OCSCC sections were made by a trained pathologist. When macrodissection was necessary for enrichment in neoplastic cells, the pathologist marked tumour areas on haematoxylin and eosin-stained tissue slides; the corresponding areas were scraped from four to five serial FFPE sections of 10 µm thickness. Adjacent mucosa from 30 of 117 FFPE specimens was analysed in samples from tumours with negative/clear margins, and the stroma immediately adjacent to the neoplastic epithelium was left as a border zone. DNA from FFPE specimens was extracted using the QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

TERT Promoter Analysis and Telomere Length Measurement

Genomic DNA amplification for *TERT* promoter region (260 bp) containing -124 C>T and -146 C>T mutation sites, as well as the SNP rs2853669 (-245 T>C), was performed exactly as previously described (21). The amplified products were purified with the Illustra ExoProStar (GE Healthcare, Buckinghamshire, UK) and sequenced on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). All samples were analysed in forward and reverse directions.

Telomere length was determined by multiplex PCR assay as previously described (22). Relative telomere length (RTL) values were calculated as telomere/single-copy gene ratio, as previously described (23).

Statistical Analysis

Differences in socio-demographic and clinical characteristics according to *TERT* promoter were tested through Fisher's exact test. For each patient, person-time at risk was computed from the date of diagnosis to the event date or the date of last follow-up, whichever came first. Events were defined as death for overall survival (OS), death or recurrence at any site for progression-free survival (PFS), local recurrence for mucosal control, and lymph node recurrence for regional failure. Analyses were truncated at 5 years. The association between *TERT* promoter and oncological outcomes was evaluated using the Kaplan-Meier method, and difference in survival probabilities was evaluated using the log-rank test (24). To account for competing risks, mucosal and regional control were evaluated using cumulative incidence, and differences according to strata were tested using Gray's test (25). The risk of unfavourable oncological outcome was evaluated using the Cox proportional hazards model (24); multivariable hazard ratios (HR), and corresponding 95% confidence intervals (CI), were calculated adjusting for gender, age, pathological lymph node status (pN), grading, surgical margins, and extracapsular invasion. For mucosal and regional control, HR were adjusted for competing risk according to Fine-Gray model (25).

RESULTS

Demographic and Clinical Characteristics of Patients

The clinical characteristics of the patients are summarized in **Table 1**. Globally, the study group had a median age of 65 years (IQR, 54-74 years) at presentation and included 81 (56.2%) male and 63 (43.8%) female patients (**Table 1**). The majority of patients were ever smoking (61.8%) and never drinking (58.3%). Tumour sub-sites within the oral cavity were as follows: 54.2% (78/144) in the tongue, 15.3% (22/144) in the floor of the mouth, 10.4% (15/144) in both the gingiva and the buccal mucosa, and 9.7% (14/144) in other sub-sites including the lip, the hard palate and the retromolar trigone. Pathological stage was T1-T2 in 99 cases (68.7%) and T3-T4 in 45 (31.3%); 47 (32.6%) of the cases had clinically positive regional lymph nodes and 97 cases (67.4%) were N0; collectively, 69 (47.9%) had advanced disease at diagnosis. Nearly 75% of tumours (104/139) showed G1-G2 grading and 25.2% (35/139) were G3. Close/positive surgical margins and positive extra-capsular spread were present in 21 (14.6%) and 16 (11.1%) cases, respectively (**Table 1**).

TERT Promoter Status

The distribution of *TERT* promoter mutations according to socio-demographic and clinical characteristics of patients are shown in **Table 1**. In the overall cohort, the promoter of *TERT* harboured mutations in 45/144 cases (31.3%). The *TERT* -124 C>T mutation was more common (29/144, 20.1%) than -146 C>T (16/144, 11.1%). These two mutations occurred in a mutually exclusive manner and with a heterozygous genotype. No mutations were observed in any of 57 adjacent available analysed mucosal specimens, 16 of which were surrounding mutated tumours. There was no statistically significant difference among analysed parameters with regard to *TERT* promoter mutation rate. We also genotyped 140 of 144 patients of our cohort for the rs2853669 SNP at -245 bp. A total of 86 patients (61.4%) carried the minor C-variant allele, for which 16 patients were homozygous and 70 were heterozygous. Fifty-four patients (38.6%) had the T/T genotype. Notably, patients with *TERT* promoter mutated tumours had a higher prevalence of the T/T genotype than patients with unmutated *TERT* promoter (p=0.0243) (**Table 1**).

Telomere Length

Measurement of RTL was obtained from 132 tumour tissues and 57 surrounding mucosal specimens. Values ranged between 0.42 and 4.42 (median 1.29) in tumours and between 0.62 and 2.93 (median 1.18) in surrounding mucosa; neither correlated with the age (data not shown). Telomere length in tumour cells and surrounding mucosa were not significantly associated with any

TABLE 1 | Distribution of 144 patients with oral cavity squamous cell carcinoma (OCSCC) according to socio-demographic and clinical characteristics, by *TERT* promoter mutational status.

		***TERT* promoter**			**Fisher exact test**
	N (%)	**unmutated N (%)**	**-124 C>T N (%)**	**-146 C>T N (%)**	
Overall	144	99 (68.8)	29 (20.1)	16 (11.1)	
Sex					
Female	63 (43.8)	41 (65.1)	12 (19.1)	10 (15.9)	p=0.3198
Male	81 (56.2)	58 (71.6)	17 (21.0)	6 (7.4)	
Age (years)					
<60	54 (37.5)	43 (79.6)	8 (14.8)	3 (5.6)	p=0.1415
60-69	39 (27.1)	27 (69.2)	8 (20.5)	4 (10.3)	
≥70	51 (35.4)	29 (56.9)	13 (25.5)	9 (17.7)	
Smoking status					
Never	55 (38.2)	41 (74.6)	9 (16.4)	5 (9.1)	p=0.5152
Ever	89 (61.8)	58 (65.2)	20 (22.5)	11 (12.4)	
Drinking status					
Never	84 (58.3)	58 (69.1)	17 (20.2)	9 (10.7)	p=1.000
Ever	60 (41.7)	41 (68.3)	12 (20.0)	7 (11.7)	
Cancer sub-site					
Tongue	78 (54.2)	55 (70.5)	16 (20.5)	7 (9.0)	p=0.6791
Floor of mouth	22 (15.3)	17 (77.3)	2 (9.1)	3 (13.4)	
Gingiva	15 (10.4)	9 (60.0)	3 (20. 0)	3 (20.0)	
Buccal mucosa	15 (10.4)	9 (60.0)	5 (33.3)	1 (6.7)	
Other	14 (9.7)	9 (64.3)	3 (21.4)	2 (14.3)	
pT					
T1-T2	99 (68.7)	66 (66.7)	20 (20.2)	13 (13.1)	p=0.5891
T3-T4	45 (31.3)	33 (73.3)	9 (20.0)	3 (6.7)	
pN					
N0	97 (67.4)	66 (68.0)	19 (19.6)	12 (12.4)	p=0.8553
N1-N3	47 (32.6)	33 (70.2)	10 (21.3)	4 (8.5)	
pStage					
I-II	75 (52.1)	49 (65.3)	16 (21.3)	10 (13.3)	p=0.5697
III-IV	69 (47.9)	50 (72.5)	13 (18.8)	6 (8.7)	
Grading[a]					
G1-G2	104 (74.8)	69 (66.4)	22 (21.2)	13 (12.5)	p=0.5676
G3	35 (25.2)	26 (74.3)	7 (20.0)	2 (5.7)	
RT					
No	93 (64.6)	61 (65.6)	20 (21.5)	12 (12.9)	p=0.5422
Yes	51 (35.4)	38 (74.5)	9 (17.7)	4 (7.8)	
CT					
No	124 (86.1)	84 (67.7)	26 (21.0)	14 (11.3)	p=0.9285
Yes	20 (13.9)	15 (75.0)	3 (15.0)	2 (10.0)	
Surgical margins					
Negative	123 (85.4)	86 (69.9)	23 (18.7)	14 (11.4)	p=0.5699
Close/Positive	21 (14.6)	13 (61.9)	6 (28.6)	2 (9.5)	
Extracapsular spread					
Negative	128 (88.9)	89 (69.5)	24 (18.8)	15 (11.7)	p=0.5367
Positive	16 (11.1)	10 (62.5)	5 (31.3)	1 (6.3)	
TERT-rs2853669[a]					
TT	54 (38.6)	31 (57.4)	12 (22.2)	11 (20.4)	**p=0.0243**
TC/CC	86 (61.4)	64 (74.4)	17 (19.8)	5 (5.8)	

[a]*The sum does not add up to total because of missing values; RT, radiotherapy; CT, chemotherapy.*
Bold values indicate p<0.05.

of the measured demographic or clinical characteristics (**Supplementary Table 1**). In keeping with our previous findings (13), we found that the mucosa adjacent to tumours harbouring *TERT* promoter mutations had significantly shorter telomeres than those in adjacent mucosa of cancers with unmutated *TERT* promoter (p=0.017) (**Figure 1A**). In particular, the surrounding mucosa adjacent to tumours with -124 C>T mutated *TERT* promoter showed the shortest telomeres (p=0.016; **Figure 1B**), despite these patients being younger [median (IQR), 64(58–70) years] than those with unmutated tumours [median (IQR), 67(48–74) years] or with tumours harbouring -146 C>T mutations [median (IQR), 75(71–80) years] (p for age=0.065; data not shown). Conversely, telomere length in tumour tissue did not significantly differ according to the mutational status of *TERT* promoter (p=0.1182) (**Figures 1A, B**).

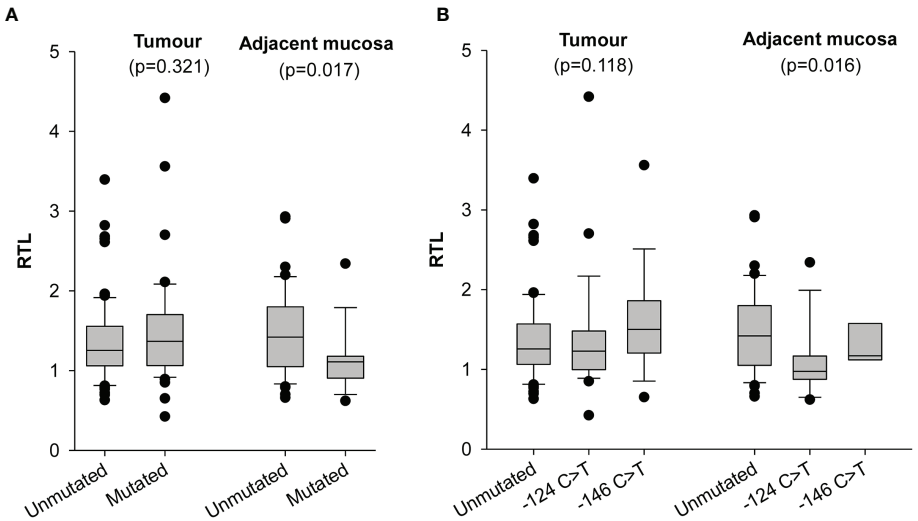


FIGURE 1 | Distribution of relative telomere length (RTL) in tumour and adjacent mucosa according to *TERT* promoter status. **(A)** samples were stratified according to absence (Unmutated) and presence of -124 C>T or -146 C>T mutations (Mutated) in the *TERT* promoter region. **(B)** samples were stratified according to *TERT* promoter status in absence (Unmutated), presence of -124 C>T and presence of -146 C>T mutations in the *TERT* promoter region.

Time-To-Event Analysis

The associations between socio-demographic and clinical characteristics of patients with clinical outcome are summarized in **Supplementary Table 2**. In a multivariate analysis adjusted for clinical variables (gender, age, pN, grading, surgical margins, and extracapsular invasion), it emerged that buccal mucosa sub-site, pathological lymph nodes, and G3 grading were significantly associated with increased risk of death (HR: 5.96, 95% CI: 1.16-30.73; p=0.0328; HR: 2.30, 95% CI: 1.12-4.75; p=0.0237; HR: 2.28, 95% CI: 1.14-4.56; p=0.0195; respectively).

In order to identify the potential impact of *TERT* promoter mutations on oncological outcome, we first investigated the association between *TERT* promoter status with PFS. Kaplan-Meier survival curve showed that the 5-year PFS for patients harbouring the -124 C>T mutation was 42.4% as opposed to 64.3% for patients without mutations, 68.2% for those harbouring the -146 C>T mutation (p=0.0069; **Figure 2B**). This association was confirmed by multivariate analysis (**Table 2**) after adjustment for clinical variables with a HR for progression of 2.71 (95% CI: 1.42-5.17; p=0.0024).

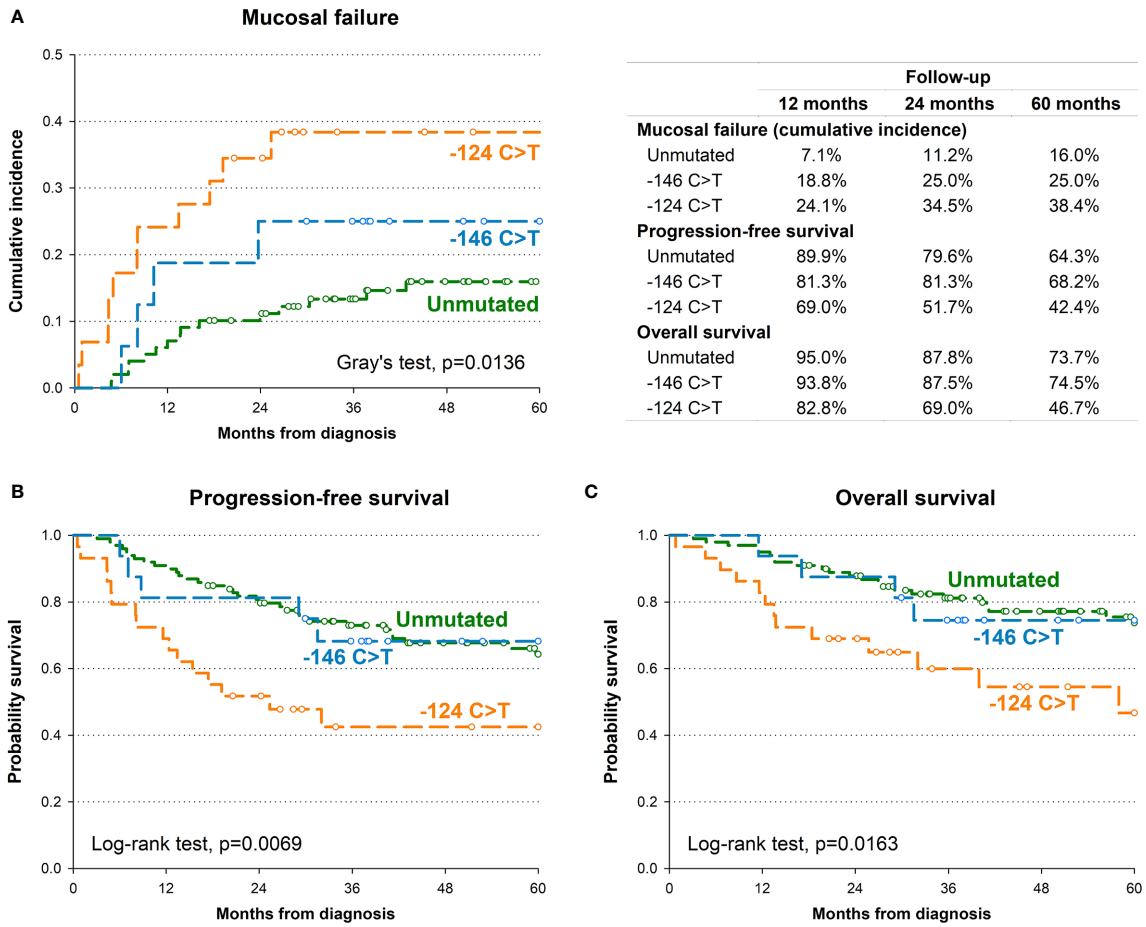
The presence of the -124 C>T mutation was also consistently associated with shorter OS, with 46.7% of patients alive after 5 years, in comparison to 73.7% and 74.5% of patients without mutations or harbouring the -146 C>T mutation, respectively (p=0.0163; **Figure 2C**). Multivariate analyses confirmed the negative effect of the -124 C>T mutation on prognosis, with a HR of death of 2.71 (95% CI: 1.30-5.66; p=0.0079) (**Table 2**). The negative impact of the -124 C>T mutation on clinical outcome was likely due to poorer mucosal control; indeed, based upon cumulative incidence estimates, patients with tumours harbouring this mutation suffered a 5-year mucosal failure rate of 38.4% in comparison to 16% and 25% in patients without mutations or harbouring the -146 C>T mutation, respectively (p=0.0136; **Figure 2A**). This association remained statistically significant in the multivariate analysis (HR: 2.75, 95% CI: 1.22-6.17; p=0.0143) (**Table 2**). These results suggest that the -124 C>T point mutation may be a risk factor for the aggressiveness of OCSCC compared to the -146 C>T mutation and unmutated *TERT* promoters which appear to be associated with a more favourable clinical outcome. Notably, the surrounding mucosa adjacent to tumours with -124 C>T mutated *TERT* promoter had the shortest telomeres (p=0.016; **Figure 1B**), and, in line with our previous studies (26, 27), adjacent mucosa with shorter telomeres (below the median value) showed a high, albeit not significant risk of tumour relapse (**Table 2**).

Kaplan-Meier survival analysis revealed that carriers of the T/T rs2853669 genotype showed significantly worse PFS (p=0.008) and OS (p=0.021) compared with C carriers (T/C+C/C genotypes) (**Supplementary Figure 1B, C**). The negative impact of the T/T genotype was confirmed in the multivariate analysis for progression (HR: 1.80, 95% CI: 1.05-3.12; p=0.0343) but not for death (HR: 1.75, 95% CI: 0.93-3.29; p=0.0837) (**Table 2**).

To evaluate if the SNP rs2853669 genotype can modulate the effect of *TERT* promoter mutations on oncological outcome, the potential role of the -124 C>T *TERT* promoter mutation as a prognostic parameter in OCSCC patients was assessed according to their rs2853669 background. Multivariate analysis revealed that the risk of mucosal failure (HR: 2.88, 95% CI: 1.01-8.25; p=0.0484), progression (HR: 5.36, 95% CI: 2.30-12.48; p<0.0001) and death (HR: 4.05, 95% CI: 1.47-11.12; p=0.0067) were significantly increased in patients with -124 C>T mutated tumours carrying the T/T genotype of the rs2853669 (**Table 2**) compared to patients without this mutation, and C carriers of the SNP.

DISCUSSION

In the present investigation, we observed that approximately one-third of OCSCC samples harboured *TERT* promoter



	Follow-up		
	12 months	24 months	60 months
Mucosal failure (cumulative incidence)			
Unmutated	7.1%	11.2%	16.0%
-146 C>T	18.8%	25.0%	25.0%
-124 C>T	24.1%	34.5%	38.4%
Progression-free survival			
Unmutated	89.9%	79.6%	64.3%
-146 C>T	81.3%	81.3%	68.2%
-124 C>T	69.0%	51.7%	42.4%
Overall survival			
Unmutated	95.0%	87.8%	73.7%
-146 C>T	93.8%	87.5%	74.5%
-124 C>T	82.8%	69.0%	46.7%

FIGURE 2 | Kaplan-Meier estimates of cumulative incidence of mucosal recurrence **(A)**, progression-free survival **(B)** and overall survival **(C)** by *TERT* promoter.

mutations with the -124 C>T mutation having a significant adverse impact on the outcome; particularly, when coexisting with the T/T genotype of rs2853669, -124 C>T mutation increased the risk of death by 4 times.

In the literature, the frequency of *TERT* promoter mutations in OCSCC varies significantly among studies ranging from 30.4 to 75% (13, 28–34). This variability could be attributable to different patient population characteristics or methodological approaches. In our cohort, we found 31.3% (45 of 144) of OCSCC samples harboured *TERT* promoter mutations, which was in line with other studies (13, 28, 30, 33, 34). In agreement with other studies on OCSCC (20, 28–32), the two mutations have different frequency, with a higher prevalence of -124 C>T (29 of 144) compared to -146 C>T (16 of 144).

With respect to oncological outcomes, an important finding emerging from this study is that the two somatic *TERT* promoter mutations displayed different behaviour. Indeed, while patients with the -124 C>T *TERT* promoter mutation had a higher risk of mucosal failure and poorer DFS and OS, patients with tumours harbouring the -146 C>T mutation had an improved clinical outcome, similar to those with unmutated *TERT* promoter. The recruitment of the transcription factor GABPA, a member of ETS family, specifically to mutant *TERT* promoters mediates long-range chromatin interaction and enrichment of active histone marks, and hence drives *TERT* transcription (35). Although both the -124 C>T and -146 C>T mutations generate identical sequences, enable binding of GABPA transcription factors, and are equally efficient in increasing *TERT* transcription *in vitro* (36), previous reports demonstrated that these mutations are not functionally identical. Indeed, a peculiar pathway of activation by non-canonical NF-κB signalling was only described for the -146 C>T mutation (37, 38). In addition, *in vivo*, the -124 C>T mutation was associated with higher *TERT* expression/telomerase activity compared to -146 C>T (39, 40). A significant body of evidence has demonstrated that high levels of tumour TERT expression and/or telomerase activity are significantly associated with aggressiveness of disease, advanced clinical stage, and poor OS and/or DFS in several types of tumours, including UADT SCC (13, 26, 27, 41). The mechanism(s) by which high TERT expression ultimately facilitates cancer progression and constitutes a prognostic factor are not completely elucidated, and seems not be attributable only to TERT's ability to maintain telomere length. Indeed, accumulating evidence suggests that

TABLE 2 | Hazard ratio (HR) and corresponding 95% confidence interval (CI)a for mucosal failure, regional failure, progression, and death according to strata of TERT promoter status, rs2853669 genotype and telomere length.

	Pts	Mucosal failure			Regional failure			Progression			Death		
		n	HR (95% CI) [b]	Wald χ^2	n	HR (95% CI)	Wald χ^2	n	HR (95% CI)	Wald χ^2	n	HR (95% CI)	Wald χ^2
TERT promoter													
unmutated	99	15	Ref		13	Ref		32	Ref		23	Ref	
-146C>T	16	4	2.46 (0.82-7.36)	p=0.1077	2	1.10 (0.21-5.73)	p=0.9127	5	1.35 (0.50-3.65)	p=0.5561	4	1.64 (0.53-5.08)	p=0.3891
-124C>T	29	11	2.75 (1.22-6.17)	**p=0.0143**	5	1.51 (0.48-4.73)	p=0.4822	16	2.71 (1.42-5.17)	**p=0.0024**	13	2.71 (1.30-5.66)	**p=0.0079**
TERT-rs2853669 (Dominant model)[c]													
CT/CC	86	15	Ref		12	Ref		25	Ref		18	Ref	
TT	54	14	1.36 (0.67-2.74)	p=0.3989	8	0.88 (0.37-2.11)	p=0.7820	28	1.80 (1.05-3.12)	**p=0.0343**	22	1.75 (0.93-3.29)	p=0.0837
TERT promoter – rs2853669													
unmut/-146C>T – CT/CC	69	10	Ref.		9	Ref.		19	Ref.		12	Ref.	
unmut/-146C>T – TT	42	8	1.33 (0.54-3.28)	p=0.5421	6	0.97 (0.35-2.69)	p=0.9476	18	1.50 (0.77-2.90)	p=0.2339	15	1.87 (0.86-4.08)	p=0.1156
-124C>T – CT/CC	17	5	2.69 (0.87-8.31)	p=0.0850	3	1.82 (0.47-7.06)	p=0.3857	6	1.79 (0.69-4.64)	p=0.2290	6	2.80 (0.98-8.00)	p=0.0539
-124C>T – TT	12	6	2.88 (1.01-8.25)	**p=0.0484**	2	1.06 (0.17-6.73)	p=0.9521	10	5.36 (2.30-12.48)	**p<0.0001**	7	4.05 (1.47-11.12)	**p=0.0067**
RTL (tumor)													
≤1.29[d]	66	15	Ref		8	Ref		25	Ref		21	Ref	
>1.29	66	13	0.92 (0.39-2.16)	p=0.8518	9	1.17 (0.36-3.87)	p=0.7946	23	1.04 (0.57-1.89)	p=0.8950	16	0.90 (0.45-1.78)	p=0.7564
RTL (surrounding mucosa)[e]													
>1.18[d]	26	3	Ref		3	Ref		5	Ref		3	Ref	
≤1.18	25	6	2.38 (0.48-11.73)	p=0.2869	1	0.07 (0.00-3.15)	p=0.1726	8	1.23 (0.37-4.14)	p=0.7366	8	2.21 (0.53-9.31)	p=0.2789

[a]*Estimated from Cox proportional hazard model, adjusting for gender, age, pN, grading, surgical margins, and extracapsular invasion.*

[b]*Adjusted for competing risk according to Fine-Gray model;*

[c]*Results for the best genetic model on OS;*

[d]*Cut-off were defined according to median value;*

[e]*Analysis restricted to patients with negative surgical margins; Pts, patients; RTL, relative telomere length.*

Bold values indicate p<0.05.

TERT may also contribute to carcinogenesis *via* telomere length-independent mechanisms, including enhancement of proliferation, resistance to apoptosis, inflammation, invasion and metastasis altogether contributing towards a more aggressive phenotype of cancer cells (10, 11, 42–50). Therefore, it is conceivable that the -124 C>T *TERT* promoter mutation, inducing higher expression of TERT in the tumour, results in the increased severity of disease as we observed in our cohort of OCSCC patients. Corroborating our results, Arantes et al. (33) found that the -124 C>T *TERT* promoter mutation was associated with increased risk of tumour relapse and death in a cohort of 88 Brazilian patients with SCC of the UADT. However, other studies in different tumour types have reported contradicting clinical effects of *TERT* promoter mutations, ranging from poorer survival associated with the -146 C>T *TERT* promoter mutation to unchanged clinical outcome (28, 29, 32, 51–54). Given that the two mutations create an identical sequence corresponding to a *de novo* binding site for ETS transcription factors, these alternative results may depend on the genetic context, including the SNP background in which *TERT* mutations arise.

For the common polymorphism rs2853669 T>C, which disrupts a pre-existing ETS2 binding site within the *TERT* core promoter, controversial clinical impacts have been reported (12, 17–20). Our study demonstrates for the first time that the rs2853669 T/T genotype influences the clinical outcome of OCSCC patients, being significantly associated with increased risk of disease progression. Importantly, the coexistence of the T/T genotype of rs2853669 and the -124 C>T *TERT* promoter mutation is associated with a significantly poorer prognosis including mucosal failure, disease progression and death. The effect of the rs2853669 SNP may be related to higher telomerase activity and *TERT* expression conferred by the T/T genotype (15) that can also additionally intensify the transactivation activity of *TERT* promoter mutations (14). Thus, we can speculate that high *TERT* levels conferred by the -124 C>T *TERT* promoter mutation and/or rs2853669 T/T genotype may promote tumour progression, probably as a consequence of the extra-telomeric non-canonical functions of telomerase. Unfortunately, we did not have enough tumour material to contemporaneously analyse *TERT* promoter status and TERT expression/activity, and further studies should be undertaken to extend and validate these findings.

A secondary finding of our study was the absent of *TERT* promoter mutations in the matched adjacent mucosa. This partly differs from a previous study by Chang et al. (29), and may be due to the reduced number of adjacent normal mucosal specimens available in our cohort. Nonetheless, the finding that metastatic and recurrent head and neck squamous cell carcinomas have more *TERT* promoter mutations compared to primary tumours (31) suggests that the acquisition of these mutations is a late event in carcinogenesis, and may explain the lack of *TERT* promoter mutations in the tumour's adjacent mucosa.

Interestingly, we confirmed that telomeres in mucosa adjacent to *TERT* promoter mutated tumours were significantly shorter than those adjacent to tumours retaining unmutated *TERT* promoter (13), and additionally we found that the mucosa adjacent to -124 C>T mutated tumours had the shortest telomeres. As critically short telomeres are a hallmark of genomic instability associated to carcinogenesis and may be considered a marker of field cancerization (26, 27), is not surprising that patients harbouring the -124 C>T *TERT* promoter mutation showed a significantly increased risk of tumour relapse. These data, for the first time, support a prognostic role for tumour relapse of the -124 C>T *TERT* promoter mutation in patients with OCSCC likely related to the very short telomeres in the mucosa surrounding the tumour in which the mutation arises.

In conclusion, we found that the -124 C>T *TERT* promoter mutation, as well as the T/T genotype of the rs2853669 SNP, may be a risk factor for the aggressiveness of OCSCC, and the coexistence of these genetic variations might represent a greater risk of adverse outcome. Supported by the fact that the clinical significance of this mutation is consistent with the biological properties of TERT, that *TERT* promoter mutations were found to stratify the prognosis in several other cancers, are easy to identify using tissue from routinely collected biopsies and address the unmet clinical need of having a validated prognostic marker for OCSCC, our observations raise the possibility that the -124 C>T *TERT* promoter mutation in combination with the SNP rs2853669 T/T genotype may serve as a valuable prognostic marker in this cancer, with the ability to guide therapeutic and follow-up strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Treviso/Belluno provinces (protocol code 346/AULSS9, March 10, 2015), Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: ADR, PBR. and SG. Methodology: SG. Statistical analysis: JP. Investigation: SG, PBR, ER, GT, LA, RDC, MR, PN, AM, VC, MT, LB, GS, EE, MMa, MS, RB, APDT, MG, MMo, and GE. Resources: ADR. Writing-original draft preparation: SG and PBR. Writing-review and editing: SG, PBR, JF, and ADR. Supervision: ADR. Project administration: PBR and SG. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.782658/full#supplementary-material

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When Everything Revolves Around Internal Carotid Artery: Analysis of Different Management Strategies in Patients With Very Advanced Cancer Involving the Skull Base

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Internal or common carotid artery encasement (CAE) is observed in almost 2-7% of head and neck cancers (HNC) and designates the tumor with the T4b category. This clinical scenario is associated with a dismal prognosis, owing to the risk for thrombosis and bleeding that usually characterizes such an advanced cancer. Standardized radiological criteria to infer invasion of the carotid artery are lacking. Complete surgical resection in the context of a multimodality treatment is supposed to offer the greatest chances of cure. Surgery can either be carotid-sparing or include carotidectomy. Data on probability of cerebrovascular and non-cerebrovascular complications, risk of carotid blowout, poor oncologic outcomes, and less-than-certain efficacy of diagnostic and interventional preventive procedures against cerebral infarction make it difficult to define surgery as the recommended option among other therapeutic strategies. Non-surgical therapies based on radiation therapy possibly combined with chemotherapy are more frequently employed in HNC with CAE. In this context, carotid blowout is the most feared complication, and its probability increases with tumor stage and cumulative radiation dose received by the vessel. The use of highly conformal radiotherapies such as intensity-modulated particle therapy might substantially improve the manageability of HNC with CAE by possibly reducing the risk of late sequalae. Despite evidence is frail, it appears

logical that a case-by-case evaluation through multidisciplinary decision making between head and neck surgeons, radiation oncologists, medical oncologists, diagnostic and interventional radiologists, and vascular surgeons are of paramount value to offer the best therapeutic solution to patients affected by HNC with CAE.

Keywords: head & neck, cancer, encasement, involvement, carotid, skull base (head and neck)

INTRODUCTION

Internal or common carotid artery encasement (CAE) by head and neck cancers (HNCs), including salivary gland and sinonasal cancers, designates the tumor with the T4b category (1). Carotid artery (CA) can be encircled, and its walls potentially invaded by the primary tumor and/or nodal metastases with extranodal extension. CAE has a low but non-negligible incidence, accounting for approximately 2–7% of advanced HNCs (2), more often in patients affected by recurrent or persistent disease. In all cases, a comprehensive, multidisciplinary plan is necessary to pursue the optimal patient-centered approach (3). This clinical scenario is generally associated with a very poor prognosis, which is determined by both the risk for fatal exsanguination when cancer erodes CA walls and the abrupt tumor progression which usually characterizes such an advanced neoplastic stage (4, 5). Moreover, CAE is usually not specified as an inclusion or stratifying criteria in trials assessing the role of non-surgical therapies.

Criteria to define CAE at imaging, mastering various therapeutic approaches for locally advanced disease with CAE, management options to prevent CA blowout (CB), as well as knowledge of potential cerebrovascular risks related to permanent CA occlusion are all contemporary aspects that should raise the interest of different physicians who deal with advanced HNCs. The present perspective will emphasize on these aspects based on the most relevant current evidence.

CAE DEFINITION CRITERIA

So far, there are no standardized radiological criteria to distinguish simple CAE from frank vessel involvement. Preoperative magnetic resonance imaging (MRI) has been demonstrated capable in predicting involvement of the CA in cases with near-circumferential encasement (6). In contrast, despite computed tomography (CT) is more commonly used to stage HNCs, its utility in predicting CA invasion has been questioned (7). In 1995, Yousem et al. analyzed 49 MRI (53 CAs) and reported that when tumor surrounded the CA for <180° or between 180–270°, no CA invasion was found at surgery. When focusing on cases with >270° encasement, CA invasion was observed in 71% of patients (6). In 2010, Pons et al. reported on 22 patients preoperatively staged through both CT and MRI. They found that combination of CA deformation, >180° encasement, and segmental obliteration of the fat separating the vessel from the adenopathy or primary tumor was highly predictive of invasion of the CA wall. On the other hand, the isolated finding of >180° encasement or fat obliteration could not reliably indicate an invasion of the CA (8). Other studies reported similar results (2, 9, 10). However, standardized and validated radiological criteria to infer CA invasion are lacking. Thus, radiological definition of CAE is challenging and not founded upon sound data. Therefore, beside counting on a head and neck imaging-trained radiologist, the multidisciplinary team should also include vascular surgeons and interventional radiologists and be equipped with the necessary resources to assess on a case-by-case basis the resectability and curability of a CA-encasing tumor.

THERAPEUTIC OPTIONS FOR PATIENTS WITH CA-ABUTTING CANCERS AND THEIR COMPLICATIONS

The therapy reported to offer the greatest chances of cure in patients with resectable HNC with CAE is surgery aimed to obtain a complete tumor resection, which can be achieved through sub-adventitial dissection or CA resection when the tumor only abuts or frankly invade/encase the vessel, respectively (**Figure 1**). The most relevant, life-threatening complication of CA-sparing surgery is CB, which has an average incidence of 3-4.5% (0-2.4% in naïve patients, 4.5-21.1% in previously irradiated patients) and mean lethality rate as high as 50% (11). In an animal study published in the 1970s, sub-adventitial dissection to peel the tumor off CA combined with infection of the surgical site have been hypothesized as being the main determinants of postoperative CB (12). Thus, one could hypothesize abutted CA to be resected irrespective of its genuine invasion by cancer, with the twofold advantage of preventing CB and providing a wider margin of resection. However, resecting the CA does not compensate the advanced stage and biological aggressiveness of HNC determining CAE. In fact, several series of HNCs, mostly represented by squamous cell carcinoma (SCC), treated through CA resection-including surgery reported a 2-year overall survival rate as low as 11.1-50.0% (13–16). On the other hand, perioperative mortality (10-25%) (17–19) and risk for cerebrovascular (12.5-33%) (13–16) and non-cerebrovascular complications (25-60%) (13, 14, 16, 20) are non-negligible in patients receiving CA resection-including surgery. Despite cerebral revascularization is supposed to reduce the incidence of cerebrovascular events (19, 21), the comparative study by Aslan et al. was unable to demonstrate a significant difference (17). Cerebral revascularization can be achieved through either CA reconstruction, which is

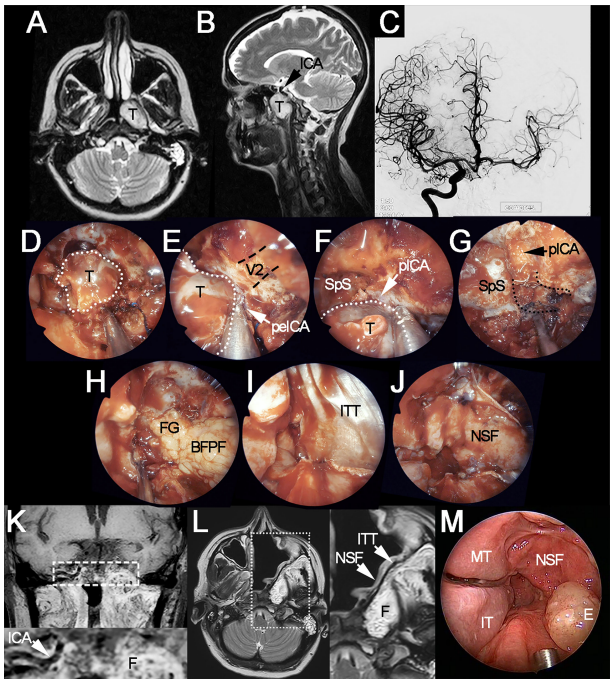

FIGURE 1 | Locally advanced polymorphous adenocarcinoma of the left nasopharyngeal wall-sphenoid sinus, treated through left extracranial-to-intracranial bypass surgery, endoscopic transnasal resection, and adjuvant intensity-modulated radiation therapy. **(A, B)** Preoperative axial and sagittal T2-weighted MRI showing the tumor (T) and its spatial relationship with the internal carotid artery (ICA). **(C)** Angiography of the temporary balloon occlusion test, which showed adequate crossflow timing but was considered as positive for ischemia due to neurological signs at sensitization through drug-induced hypotension. **(D)** Endoscopic appearance of the tumor (white dotted line) after sphenoidotomy, ethmoidectomy, and medial maxillectomy. **(E, F)** Intraoperative evaluation confirmed the presence of tight adhesion to the caudal paraclival (pICA) and medial petrous (peICA) tracts of the internal carotid artery. **(G)** Endoscopic view of the surgical field following carotidectomy, occlusion coils can be sees as emerging from the distal stump of the vessel (i.e., cranial paraclival tract). Clearance of the carotid canal (black dotted lines) and petroclival junction have been performed. **(H–J)** Reconstruction of the skull base defect through the right buccal fat pad flap (BFPF), fat graft (FG), iliotibial tract graft (ITT), and the right nasoseptal flap (NSF). **(K)** Coronal T1-weighted, fat-saturated, contrast-enhanced MRI acquired 10 months after surgery (7 months after completion of adjuvant radiation therapy). White dashed rectangle indicates the position of magnification at the bottom of the image, which shows the right internal carotid artery and enhancing fat **(F)** in the position of the left carotid canal and petroclival junction. **(L)** T2-weighted MRI acquired 10 months after surgery. White dotted rectangle indicates the position of magnification on the right of the image, which shows the layers of the reconstruction. **(M)** Endoscopic appearance of the surgical site 10 months after surgery. V2, position of the maxillary nerve (black dashed lines indicate the trajectory of the nerve); E, mucosal edema; IT, inferior turbinate; MT, middle turbinate; SpS, sphenoid sinus.

technically feasible when common CA and/or extracranial (i.e., parapharyngeal) internal CA are resected, or bypass surgery, which consists of creating a communication between a donor arterial system, such as the external carotid one, and the cerebral vascularization (e.g., to the middle cerebral artery) *via* an interpositional vascular graft. Moreover, prior to indicate CA resection without cerebral revascularization in a patient tolerating a temporary balloon occlusion test, one should consider that the rate of delayed cerebrovascular events in patients with negative occlusion test accounts for 15-22% (22–24). Of note, more than one study demonstrated that morbidity and mortality in patients receiving CA resection-including surgery had a decreasing trend after the 1990s (18, 19). These data taken altogether suggest that only meticulous selection of patients and minimization of surgical morbidity, which cannot prescind from involvement of a neurologist and neurosurgeon in the multidisciplinary team, could lead CA resection-including surgery to be a valuable therapeutic option for some patients with CAE-determining HNC. As an example of the need to accurately select patients, Yokoyama et al. reported a series of 10 patients receiving CA resection and reconstruction through a superficial femoral vein graft: the 5-year overall survival rates of patients affected by SCC and non-SCC cancers were <20% *versus* 100%, respectively (25). These data witness that histology represents one of the factors to consider when CA resection-including surgery is proposed.

Nonsurgical modalities, mainly represented by photon-based radiotherapy (RT), delivered either alone or in combination with chemotherapy (CRT), are aimed at avoiding complications of CA resection. An HNC may be labelled as "unresectable" either because genuinely unsuitable for a wide-margin resection (i.e., invasion of the skull base, nasopharynx, prevertebral space or cervical spine, fixation of nodes, massive bilateral nodal involvement) or due to the estimated unfavorable balance between risks of surgery and its potential benefits from an oncologic standpoint. Over one-third of these patients are

usually treated by neoadjuvant chemotherapy followed by CRT (26–29). However, there are few data regarding the outcome of nonsurgical treatments in patients affected by HNC with CAE. Roh et al. reported a cohort of patients with CA-invading HNC: the median survival was 16.5 months in patients treated with surgery (n=11) with or without reconstruction or ligation of the CA, possibly combined with (neo)adjuvant (C)RT, 11.5 months for patients receiving definitive (C)RT (n=6), and 3 months for those treated palliatively (n=6) ($p<0.05$). CA was not occluded in patients receiving RT, some of them undergoing a temporary balloon occlusion test prior to treatment (5). In addition, no separate outcome analysis was performed for naïve and recurrent patients. Manzoor et al. also reviewed the outcomes of 44 consecutive *de novo* and recurrent HNCs patients with CA involvement. Survival outcome was not significantly different between patients treated with definitive CRT and surgery with or without postoperative RT ($p=0.47$), although a trend was found in favor of CRT, possibly because of the treatment-naïve nature of these patients. Of note, imaging was assessed in 7/8 patients treated with radical CRT, and all had near-total circumferential CAE (30).

No data on CB events were reported in non-surgically treated patients in these two latter series (5, 30). However, CRT can determine the obliteration of the carotid vasa vasorum, leading to fibrosis of the adventitia and subsequent weakening of the arterial wall (31). Indeed, in a study on 1072 patients receiving CRT with conventional fractionation for HNC, the cumulative incidence of CB increased stepwise from 1.4% to 6.1% considering T1 to T4 cancers, suggesting that locally advanced tumors are associated with a higher risk of CB (32). The overall incidence of CB further increases in patients undergoing re-irradiation for HNC (11, 33), with CAs receiving a cumulative dose of 120 Gy or higher blowing out in 25% of cases within 1 year (34). Of note, the highest CB rates published were in patients affected by recurrent nasopharyngeal cancer re-irradiated through hypofractionated stereotactic RT. These data pose a considerable dilemma to the radiation oncologist, who is forced to either delivering a suboptimal dose or putting the patient at risk of CB, particularly in the re-irradiation setting. This concern could be tempered when delivering high precision RT, like protons and carbon ions. High-linear energy transfer carbon ions RT (CIRT) has recently entered the clinical practice. It enables dose escalation due to specific ions physical properties (allowing highly conformal dose distributions) and offers superior relative biological effectiveness by at least a 2-3-fold factor in comparison to conventional RT (35). There is some evidence that radioresistant HNCs and skull base tumors, such as adenoid cystic carcinoma, may benefit from CIRT, usually using hypofractionated regimen, in terms of outcome and safety. This is particularly true in inoperable/unresectable tumors, macroscopic residues, and recurrences (36–38). So far, there are scant data on the occurrence of vascular complications after CIRT. Jensen et al. reported a CB incidence of 3.8% in 52 patients receiving CIRT-based re-irradiation for recurrent adenoid cystic carcinoma. One-year local control and overall survival were 70.3% (2-year estimate: 47.4%) and 81.8% (2-year estimate: 63.3%), respectively, which is higher compared to conventional RT (39). In another paper, only 1/229 patients re-treated with CIRT had a CB. This was a patient with a recurrent adenoid cystic carcinoma of the right base of the skull already treated with 2 courses of RT. The patient recovered quickly from a post-interventional stroke and survived for 9 months after CB. Median local progression-free survival after CIRT was 24.2 months and the median overall survival 26.1 months (40). Neither of these two studies analyzed the radiological relationship between CA and the tumor, nor was the possibility to stent or occlude CA before starting re-treatment discussed. The latter strategy should be considered in view of the potential benefits in terms of local control and survival in certain histologies candidates to CIRT (**Figure 2**).

CONCLUSIONS

Patients with naïve or recurrent advanced HNC with CAE may still have a chance to be cured if treated with modern surgical and RT techniques. In particular, this should be taken into account in young patients with a relatively indolent disease, who may potentially have a relatively long life expectancy. In this clinical scenario, a careful evaluation of the available management strategies to secure CA should be put in place to achieve the best oncological results while minimizing the risk of

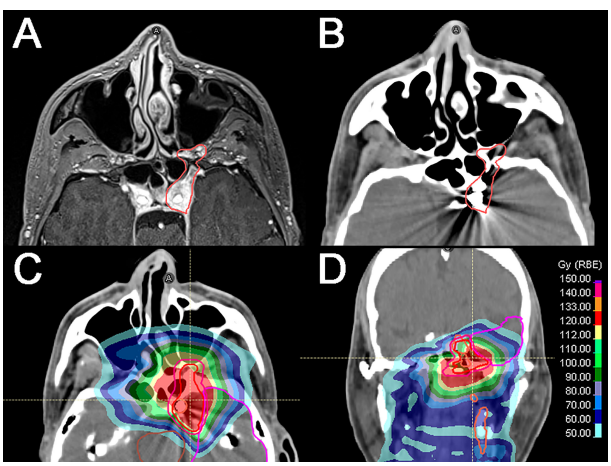

FIGURE 2 | Locally recurrent nasopharyngeal squamous cell carcinoma diagnosed 5 years after the initial diagnosis (cT3N1). **(A)** Axial T1-weighted, fat-saturated, contrast-enhanced image shows the recurrence (*red line*) extending through the left foramen rotundum and vidian canal and involving the foramen ovale and cavernous sinus. Left cavernous internal carotid artery (ICA) is encased by the tumor. **(B)** Axial CT simulation image showing the tumor (red line) and the left ICA occluded with endovascular coils following a well-tolerated temporary balloon occlusion test. **(C, D)** CT axial and coronal images showing the tumor and the cumulative dose distribution according to the primary photon RT plan and the definitive re-irradiation through intensity-modulated proton therapy (54 GyE). Isodose levels are represented by different colors.

cerebrovascular and non-cerebrovascular sequelae. Precise analysis of tumor extension, adequate treatment planning, and proper counseling should save patients an ineffective invasive treatment, such as an unintentional R2 surgery, and the risk of dangerous and potentially life-threatening complications, such as CB. These prerequisites are best fulfilled in tertiary referral centers, where the multidisciplinary team can handle very advanced cancers with CAE by exploiting the available strategies and customizing treatment based on special characteristics of the single case. It is authors' opinion that prospective studies are needed to objectively assess the risk-benefit ratio of CA securing strategies (e.g., stenting or occlusion) that are adopted to deliver the locoregional treatment of HNC with CAE.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Authors contributed as follows: conception (EO, MF, EL, LP, MBe, BV, MBo, VR, AS, LL, and PN). Perspective design (EO and MF). Paper draft (EO and MF). Draft correction (EL, LP, MBe, BV, MBo, VR, and AS). Supervision (LL and PN). All authors contributed to the article and approved the submitted version.

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Leukoplakia: An Invasive Cancer Hidden within the Vocal Folds. A Multivariate Analysis of Risk Factors

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Introduction: Discerning the preoperative nature of vocal fold leukoplakia (VFL) with a substantial degree of certainty is fundamental, seeing that the histological diagnosis of VFL includes a wide spectrum of pathology and there is no consensus on an appropriate treatment strategy or frequency of surveillance. The goal of our study was to establish a clear schedule of the diagnostics and decision-making in which the timing and necessity of surgical intervention are crucial to not miss this cancer hidden underneath the white plaque.

Material and Methods: We define a schedule as a combination of procedures (white light and Narrow Band Imaging diagnostic tools), methods of evaluating the results (a combination of multiple image classifications in white light and Narrow Band Imaging), and taking into account patient-related risk factors, precise lesion location, and morphology. A total number of 259 patients with 296 vocal folds affected by leukoplakia were enrolled in the study. All patients were assessed for three classifications, in detail according to Ni 2019 and ELS 2015 for Narrow Band Imaging and according to Chen 2019 for white light. In 41 of the 296 folds (13.9%), the VFL specimens in the final histology revealed invasive cancer. We compared the results from the classifications to the final histology results.

Results: The results showed that the classifications and evaluations of the involvement of anterior commissure improve the clinical utility of these classifications and showed improved diagnostic performance. The AUC of this model was the highest (0.973) with the highest sensitivity, specificity, PPV, and NPV (90.2%, 89%, 56.9%, and 98.3%, respectively).

Conclusion: The schedule that combines white light and Narrow Band Imaging, with a combination of the two classifications, improves the specificity and predictive value, especially of anterior commissure involvement.

Keywords: vocal fold leukoplakia, narrow band imaging (NBI), glottic cancer, white light, anterior commissure (AC)

INTRODUCTION

Vocal fold leukoplakia (VFL) is a clinical term that describes a white patch or plaque resulting from epithelial parakeratosis, but does not specify what is hidden within the lesion. The histological diagnosis of VFL includes a wide spectrum of pathology, through stages of dysplasia to invasive cancer (1, 2). Additionally, there is no consensus on the threshold for surgical intervention, appropriate treatment strategy (wait and see policy, sampling, or excisional surgery), or the frequency of surveillance (3, 4). Therapeutic decisions balance high-quality voice preservation and oncological safety, thus caution is taken while referring a patient for surgical treatment and techniques are chosen to eliminate a superficial lesion while preserving the underlying vibratory mucosa. Some authors claim that a 'wait and see' approach is appropriate for smooth lesions, while some believe that more aggressive treatment should be performed (5, 6). In any event, the most popular treatment of VFL is surgery, even though in 50% of specimens, the final histology does not show dysplasia or cancer (7, 8). In aiming to resolve these discrepancies, diagnostic methods and recommendations such as the schedule for VFL management should be of utmost importance (9).

White light (WL) laryngoscopy is the first line and the most important diagnostic tool in the assessment of the pathologies of the larynx, including VFL (10, 11). However, it has limitations, especially in the assessment of underlying vascularization, which can be resolved by using additional light settings. Narrow Band Imaging (NBI) is a well-established bioendoscopic technique that uses filtered wavelengths to enhance the microvascular pattern and its alterations are associated with preneoplastic and neoplastic transformation of the upper aerodigestive tract mucosa (12–16). NBI using blue light (wavelength peak of 400 to 430 nm) and green light (wavelength peak of 515 to 555 nm), which correspond to the absorption peaks of hemoglobin (12), enhances the physicians' chances to detect and delineate the suspicious mucosal lesions in a non-invasive way, and is thus beneficial to the diagnosis of a variety of benign and malignant lesions (14). NBI proved to be a useful diagnostic tool for the assessment of laryngeal leukoplakia; however, there is no clear place or recommendations for using NBI in preoperative assessments of VFL (17, 18) or establishing this method as the guidance for management (19)

The researchers' hypothesis assumes the confirmation of a simple diagnostic schedule by using WL and NBI for patients with laryngeal leukoplakia in stratifying cancer risk to avoid false negative or false positive histological findings and vocal fold damage.

Therefore, the primary aim of this paper is to present the gathered and analyzed data to express which factors increase the risk of malignant transformation inside leukoplakia to avoid under- or overtreatment.

The second goal of our study was to confirm the usefulness of a clear diagnostic and decision-making schedule in which the timing and need for surgical intervention are crucial to not miss this cancer hidden underneath the white plaque.

MATERIAL AND METHODS

Study Population

A prospective study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Lodz (RNN/225/19/KE, 9 April 2019) at the Poznan and Lodz Otolaryngology Tertiary Referral University Departments, between January 2015 and March 2020.

259 consecutive patients were enrolled – 212 men (81.85% of the cohort), 47 women (18.15%). The mean age was 62.08 years for males, and 61.2 years for females. VFL was diagnosed on 296 vocal folds.

The inclusion criteria were: a diagnosis of vocal fold leukoplakia confirmed by endoscopic evaluation under white light and NBI, no prior vocal fold-related medical intervention or procedures. Exclusion criteria were: other benign lesions (cysts, polyps, Reinke's edema, or papilloma) in endoscopic evaluation; a history of laryngeal surgery, trauma, or intubation; a history of radiotherapy and chemotherapy for head and neck, as well as a lack of written consent from the patient and the presence of changes in endoscopic evaluation suggestive of an advanced neoplasm.

Clinical Diagnostic Work-Up

All patients were assessed with a flexible transnasal video endoscope (Olympus Medical Systems Corporation, Tokyo, Japan) by means of WL and NBI. In the first step, the VFL in white light was observed regarding the texture, color, size, redness, symmetry, and thickness (according to the Chen 2019 classification). A fingertip control switch on the endoscope then changed the view to the NBI mode, and the vascular pattern was assessed, with close attention paid to the presence of intraepithelial papillary capillary loops (IPCLs) (according to the Ni 2019 (20) and European Laryngological Society (ELS) 2015 classifications).

Two independent physicians from each institution (JJ, HK, WP, JM), with at least 3 years of experience in the use of NBI, independently assessed each patient. For high-risk leukoplakia in the Chen classification, the cut-off point was 3 (elevated and rough leukoplakia); in the Ni classification, the cut-off point was 5 (IPCLs outside leukoplakia); and in the ELS classification, the cut-off point was 2 (perpendicular vessels).

Based on these classifications, the patients were qualified as at low or high risk of leukoplakia. The combination of these three classifications and cut-off points in the preoperative assessment of VFL was described by Pietruszewska et al. (2021) (9). Therefore, we used this scoring methodology for the leukoplakia image in WL and NBI.

In addition to the WL and NBI classification scores, additional variables were the patient's age, sex, smoking habits, uni- or bilateral lesions, anterior commissure (AC) involvement, and the uni- or multifocal nature of the lesions.

All patients underwent transoral microsurgery by cold instrumentation, and specimens were sent for final histology. A frozen section was not performed due to the very small size of the specimens, which encompassed only the epithelial layer.

Histopathological diagnosis was performed according to the WHO classification system to classify the resected tissue as low-risk or high-risk dysplasia (21). The main predictive variables taken into consideration were the vascular pattern according to the Ni classification (2019), the ELS classification (2015), and the morphological characteristics according to the Chen classification (2019), along with the final histological findings.

Statistical Methodology

Data were stored in a computer-based filing system and reported as absolute and relative frequencies. Statistical analysis was performed in STATISTICA 13.1 Software (Dell, USA). The cut-off values for classifications with more than two degrees were established based on the receiver operating characteristic (ROC) curve analysis, and the highest Youden's index in each classification determined the proposed cut-off value, as per Fluss et al. (22). To assess the diagnostic performance of the clinical classifications, the measures of occurrence (sensitivity, specificity, and accuracy) and the possibility of discriminating (positive and negative predictive values) for clinical classifications of WL and NBI endoscopy were calculated per the determined cut-off values. ROC curve comparisons for the analyzed classifications are presented in **Figure 1**. Odds Ratio's for particular types of non-dychotomized classification systems by Ni 2019 and Chen 2019 are provided as **Supplementary Material** (**Supplementary Figures 1**, **2**).

Multivariate Analysis

Multivariate analysis was performed by means of logistic regression, the results of which are presented in a format suggested by Peng et al. (23) To perform logistic regression analysis on the probability of developing invasive cancer from leukoplakia, the following leukoplakia and epidemiological characteristics were analyzed in a univariate analysis: age, gender, leukoplakia localization, the focality of the leukoplakia, involvement of the anterior commissure, history of smoking, and the results of clinical classifications in WL and NBI. All variables were analyzed using the Likelihood Ratio (LR) test. The test was considered statistically significant if the p-value was < 0.05. The LR test results and the inclusion of variables for further multivariate analysis are summarised in **Table 1**.

The variables were checked for interactions and linearity of predictors using the LR test. No interactions between variables were detected. The linearity test result for age was $p = 0.59$; therefore, the variable was linear. A mixed effects logistic regression model was constructed to model a binary outcome of 0 (not developing invasive cancer) or 1 (developing invasive cancer under the leukoplakia). The equation to predict the probability of developing invasive cancer under the leukoplakia is presented below:

$$\text{Predicted logit} = (-7,753 + (1,628) \cdot \textit{Anterior Commisure Involvement} + (2,496) \cdot \textit{WL} + (3,742) \cdot \textit{ELS})$$

The model's predictive ability was validated using the V-fold cross-validation method with 10 subsets of data. The model's goodness of fit was evaluated with the Hosmer-Lemeshow test (p=0.623). The discrimination curves for the regression model and the V-fold cross-validation are depicted in **Figure 2**. The results of the multivariate analysis are summarised in **Table 2**. The predictive and diagnostic capabilities of the model were evaluated using ROC analysis, which is shown in **Table 3**.

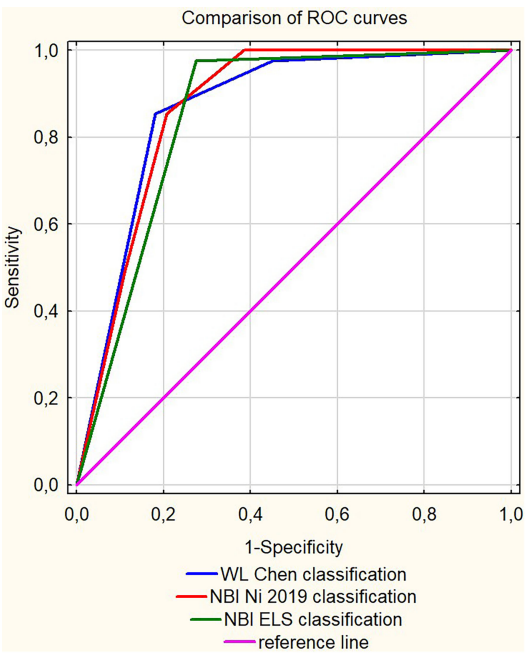

FIGURE 1 | The receiver operating characteristic (ROC) curves comparison for the analyzed clinical classifications.

RESULTS

Invasive cancer was confirmed in 41 (13.6%) out of the 296 VFL specimens in the final histology. Among this group, the mean age was 64.24, with 5 (12.2%) females and 36 (87.8%) males. 38 (92.7%) of the 41 patients were heavy smokers, and 23 (56.1%) were heavy drinkers. Taking into consideration the precise localization of the plaque carrying cancer, all 41 (100%) specimens were unilateral, but in 16 (39%), the lesions spread in the AC. Taking into account the number of VFL points, 24 (58.5%) presented unifocal and 17 (41.5%) multifocal. The complete characteristics of the study group divided by histopathological results Are available in the **Supplementary Material**.

Figure 3. Leukoplakia in WL (acc. to Chen type II) and NBI (acc. to Ni type V, acc. to ELS type II).

Using the Chen classification, out of the total number of 41 lesions only 1 (2.4%) was assessed as type 1 (flat and smooth), 5 (12.2%) as type 2 (elevated and smooth), and 35 (85.4%) as type 3 (rough).

TABLE 1 | Summary of univariate analysis conducted as the first step towards formulating a logistic regression model.

Variable	P (LR) Univariate analysis	Included in further analysis	OR (95%CI)
Age	0.068	No	1.033 (0.997;1.071)
Age categorized	0.017	Yes	2.53 (1.124;5.708)
0: below 60.			
1: 60 and older			
Gender	0.196	No	1.844 (0.069;4.933)
0: male			
1: female			
Leukoplakia localization	<0.001	Yes	0.03 (0; 0.42)[#]
0: unilateral			
1: bilateral			
Focality of leukoplakia	0.877	No	0.49 (0.486;1.852)
0: unifocal			
1: multifocal			
Anterior commissure involvement	0.049	Yes	1.91 (1.03;3.802)
0: not involved			
1: involved			
Smoking	0.014	Yes	3.646 (1.085;12.25)
0: non-smoker			
1: current or former smoker			
WL classification acc. to Chen's 2019 classification	<0.001	Yes	26.504 (10.531;66.705)
0: stage I and II			
1: stage III			
NBI ELS classification	<0.001	Yes	105.71 (14.259;783.725)
0: longitudinal vessels			
1: perpendicular vessels			
NBI Ni's 2019 classification	<0.001	Yes	22.23 (8.884;55.641)
0: stage to IV			
1: stage V and VI			

Haldane-Anscombe correction was applied to account for cells with 0 cases.

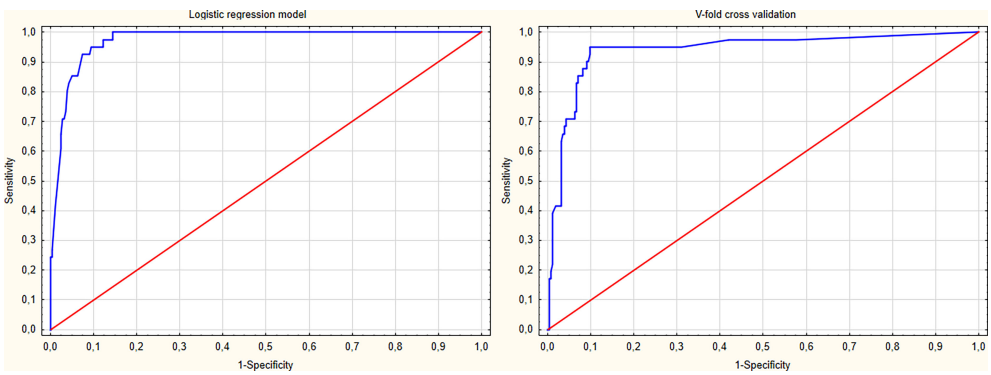

FIGURE 2 | The receiver operating characteristic (ROC) curves of the proposed logistic regression model and the v-fold cross validation of the model.

Using the Ni classification, 2 (4.9%) lesions were assessed as type 3 (IPCLs can be seen at the surface of the vocal fold mucosa surrounding the leukoplakia without clear boundaries), 4 (9.8%) as type 4 (IPCLs can be observed on the surface of the white plaque), 15 (36.6%) as type 5 (IPCLs can be seen on the vocal fold mucosa outside the leukoplakia with regular boundaries), and 20 (48.8%) as type 6 (IPCLs can be seen on the vocal fold mucosa outside the leukoplakia and on the surface of the plaque).

Using the ELS classification: 1 (0.4%) of the 41 had type 1 (longitudinal vascular pattern) and 40 (97.5%) had type 2 (perpendicular vascular pattern) leukoplakia.

Univariate Analysis

Univariate analysis revealed that bilateral vocal fold changes were 0.03 times less likely to present as invasive cancer ($p < 0.001$). However, this observation was not included in the regression model because there were no cases of invasive cancer present in bilateral VFL.

Considering age as a continuous variable, the LR test results were close to statistical significance (p=0.068). However, in order to include age in the multivariate analysis, age was dichotomized using the Weight of Effect (WOE), and similar WOE values were combined into two categories: patients up to 60 years and older

TABLE 2 | Summary of multivariate analysis using logistic regression, including the parameters for the regression model and goodness of fit test results.

Predictor	β	*SE*β	Wald's χ^2	*df*	p	e^{β} (Odds Ratio) [95%CI]
Constant	-7.753	3.373	5.285	1	0.022	NA
Anterior commissure involvement 0: not involved 1: involved	1.628	0.684	5.672	1	0.017	5.094 [1.334;19.452]
WL Chen's 2019 classification 0: stage I and II 1: stage III	2.496	0.687	13.203	1	<0.001	12.134 (3.157;46.638)
NBI ELS classification 0: longitudinal vessels 1: perpendicular vessels	3.742	1.144	10.702	1	0.001	42.183 (4.482;397.015)

NA, not applicable.

TABLE 3 | Diagnostic performance of the analyzed endoscopic classifications and proposed cut-off values for determining the risk of cancer development under vocal fold leukoplakia.

Clinical classification	Youden's index	Proposed cut-off value	AUC	Sensitivity	Specificity	PPV	NPV
WL Chen's 2019 classification	0.67	3	0.867	85.4%	82%	43.2%	97.2%
Narrow Band Imaging ELS classification	0.7	1	0.851	97.6%	72.5%	36.4%	99.5%
NBI Ni's 2019 classification	0.65	5	0.823	85.4%	79.2%	39.8%	97.1%
Proposed logistic regression model			0.973	92.7%	92.9%	67.9%	98.8%

AUC, Area under curve; PPV, positive predictive value; NPV, negative predictive value; WL, white light; NBI, narrow band imaging

than 60. In this case, people over 60 were 2.53 times more likely to develop cancer under the leukoplakia (p=0.017). A history of smoking (present or former tobacco users) was associated with a 3.646 times increase in the probability of developing cancer. Neither gender nor the focality of the leukoplakia (multifocal or unifocal plaques on one vocal fold) was associated with statistically significant odds of malignant transformation under VFL.

Multivariate Analysis

In the multivariate analysis using a logistic regression model, VFL involving the anterior commissure was 5.094 times more likely to present with invasive cancer (p=0.017). The plaques that were rough and elevated (stage III in WL) were also associated with significantly higher odds of developing cancer (OR 12.124, p <0.001). The presence of a perpendicular blood vessel pattern surrounding the plaque (ELS grade 2) was associated with a 42.183 times higher risk of malignant transformation (p <0.001). The results of the multivariate analysis are presented in **Table 2**.

Diagnostic Performance

To evaluate the diagnostic performance of different clinical classifications regarding the risk of malignant transformation in clinical leukoplakia, we compared the AUC (Area Under Curve) values, sensitivity, specificity, and positive and negative predictive values. Among the clinical classifications proposed by different authors, the highest AUC of 0.867 in the ROC analysis was reported for WL evaluation according to Chen et al. (2019), with a sensitivity of 85.4% and specificity of 82%, and Negative Predictive Value (NPV) at 97.2%; however, Positive Predictive Value (PPV) was low at 43.2%.

The evaluation of leukoplakia with NBI utilizing the Ni 2019 classification did not significantly improve the diagnostic performance, with specificity being lower than WL (79.2% *vs.* 82%), sensitivity at the same level, similar to NPV of 97.1%, and an even lower PPV (39.8% *vs.* 43.2%).

Introducing the ELS classification to the NBI endoscopy shows better results than the Ni 2019 classification, with slightly higher AUC at 0.851, higher sensitivity (97.6% *vs.* 85.4%) but with lower specificity (72.5% *vs.* 79.2%).

The diagnostic algorithm derived from the logistic regression analysis, which included evaluation of a patient in NBI, WL, and evaluation of anterior commissure involvement, improves the clinical utility of these classifications and shows improved diagnostic performance. The AUC of this model was the highest (0.973), with the highest sensitivity, specificity, PPV, and NPV (90.2%, 89%, 56.9%, and 98.3%, respectively).

DISCUSSION

In the presented study, we have shown the tactics to resolve the clinical challenges that laryngologists face balancing therapeutic decisions in vocal fold leukoplakia. The philosophy of managing such patients is to consider both oncological efficacy and functional outcomes. Thus, our hypothesis assumed that a combination of procedures (WL and NBI), scoring systems (combining multiple image classifications in WL and NBI), patient-dependent variables, and the precise location of the plaque contribute to the ability to not miss the cancer.

Since its first introduction in the late 1990s, the use of NBI has considerably contributed to physicians' ability to non-invasively detect and delineate the suspicious mucosal lesions (19). Nowadays, NBI is a mainstay of diagnostics, although in VFL,

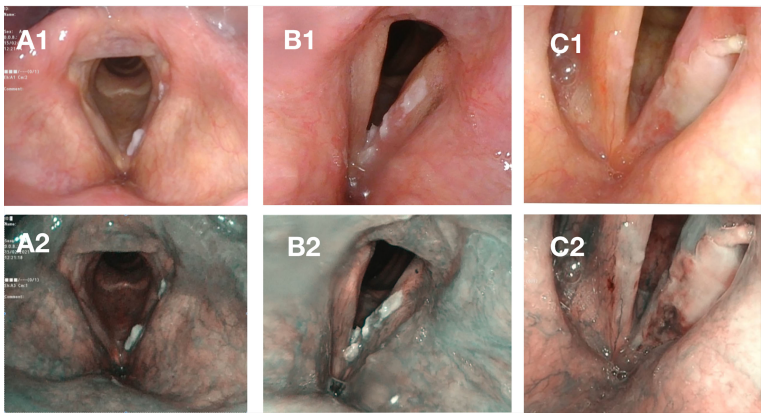

FIGURE 3 | Case A – elevated and smooth leukoplakia seen in white light [WL] **(A1)** and Narrow Band Imaging [NBI] **(A2)** with no evidence of neoplastic proliferation and only low-grade dysplasia in histopathological examination. Case B – elevated and rough leukoplakia in WL **(B1)** with pathologic vessels visible in the periphery of the plaque in NBI **(B2)**, high-grade dysplasia in histopathological examination. Case C – elevated and rough leukoplakia **(C1)** with pathologic vessels visible in the periphery of leukoplakia on the frontal part of the left vocal fold in NBI **(C2)** with invasive carcinoma in histopathological examination.

it has some limitations connected with the umbrella effect due to thick layers of keratin covering the vascular pattern. Nevertheless, in recent years, this impediment has been overcome through the assessment of the vascularization outside the plaque, and a number of studies have proved that NBI can be applied with success in VFL (24–26). But the problem remains in standardizing cut-off points and translating the NBI findings into firm indications for surgery or, on the contrary, to continue with watchful waiting.

Different classifications in recent decades (12, 16–19) were proposed. However, there is still the need for a common, unified schedule to be shared among clinicians to describe WL findings and NBI-enhanced vascular patterns to distinguish the nature of VFL.

In 2015, ELS introduced a simplified classification for vascular changes of the vocal folds divided between longitudinal and perpendicular vascular changes. The perpendicular changes present as intraepithelial papillary capillary loops (IPCLs) that are connected with laryngeal papillomatosis, precancerous, and cancerous lesions (27). Many authors confirmed the high accuracy of ELS classification in determining between benign and malignant lesions (28–30). In 2019, Ni et al. improved the classification from 2011, adding another six types that cover vocal fold leukoplakia. Both classifications are based on the morphological changes present in laryngeal IPCLs to distinguish them between benign and malignant lesions (31). In the new classification, the focality is on the presence of perpendicular vessels outside or on the surface of the plaque, which suggests a malignant lesion. The pathological vessels seen in NBI are visualized as brown dots of different sizes and are twisted, earthworm-like. The accuracy of this classification in the assessment of VFL was 90.8%, which is significantly better than that of conventional WL endoscopy (70%) (31, 32).

Our paper presented similar results. Using NBI in laryngeal leukoplakia diagnosis revealed high accuracy, especially when using the ELS classification. Specifically, the accuracy was 93.9%, according to ELS, and 85.1%, according to the NI 2019 classification. The combined use of contact endoscopy (CE) and NBI has already been suggested for visualizing specific vascular changes indicative of glottic neoplasia (33). However, inter-rater reliability and agreement in three classification systems proved to be the best for vascular changes by the ELS and was significantly higher than those by Ni et al. and Puxeddu et al. (33).

Other than NBI endoscopy, systems like the Storz Professional Image Enhancement System have also been increasingly used in patients with suspected lesions of the larynx and hypopharynx (34). Both methods are comparable in the detection and analysis of superficial neoangiogenesis, and both methods are efficient in observing epithelial and subepithelial microvascular irregularities and pathologies, but NBI has been more popularized (35). A conventional laryngoscope using white light plays the main role in the decision-making process concerning VFL in the majority of laryngology departments. In 2019, Chen et al. proposed a new WL classification for VFL connected with the morphological features of the lesions, and distinguished three types of plaque: flat and smooth, elevated and smooth, and rough. They showed a high correlation between the morphological features of the laryngeal leukoplakia and their final histology result (19).

Another issue is the precise location of the lesion in the glottis area. It is known that the anterior commissure raises oncological concern because it represents a weak point with regard to tumor spread (36, 37). There are different degrees of AC involvement (36), but this stratification is concerned with tumor infiltration and not with the location of the leukoplakia. The very small distance between anterior commissure mucosa and thyroid cartilage, and the lack of perichondrium or periosteum in the AC area promote the spread of cancer, even in early invasion (38, 39). This should not affect the risk of cancer in VFL, which affects

the superficial epithelial layers. Nevertheless, the AC is a site susceptible to the influence of tobacco smoke carcinogens on the epithelium.

Thus, we wanted to check whether the plaque in the AC location should be treated more suspiciously. We confirmed that this area should receive greater attention because the odds of cancer developing under a leukoplakia plaque in lesions involving AC were 5.094 times higher than those not involving the anterior commissure.

In this paper, we stabilize the schedule to recognize cancer under VFL plaque on a large, multicenter group of patients. The flowchart used in this research followed the schedule published by Pietruszewska et al., but we add an additional variable, AC involvement, which in our opinion, is crucial for cancer prediction. The algorithm includes a scheme of action based on a physical examination: morphology of plaque in WL according to the Chen classification, the vascular pattern in NBI scored according to the ELS classification, and the precise localization of the plaque.

Our results showed a high correlation between both the ELS and Chen classifications and the pathological outcomes, which is comparable to the results of Lin et al. (40) and Lu et al. (41). Therefore, we believe that the combined classifications of ELS and Chen, and the addition of the focus on AC involvement play a basic role in the diagnosis of laryngeal leukoplakia, especially in determining and distinguishing between those that are benign and those that are malignant. These findings have clinical importance for initial VFL diagnosis, directing patients for surgery, and routine endoscopic surveillance.

This study has some strengths and limitations. The strong points of this study include the creation of a new schedule for VFL diagnostics based on WL and NBI with regard to AC location in one of the biggest clinical groups of VFL. The combination of methods applied according to the proposed schedule proved effective in distinguishing cancer underneath the leukoplakia and gives direct suggestions for treatment options. However, there are still several weaknesses in the study. The main issue concerns the prevalence of NBI in laryngology departments; this method is not as popular as white light endoscopy, and additionally, the learning curve of NBI is quite long.

CONCLUSIONS

The ability to detect invasive cancer under leukoplakia remains a diagnostic challenge. An algorithm that combines WL and NBI, and the combination of two classifications, improves the specificity and positive predictive value, especially in anterior commissure involvement.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Komisja Bioetyczna przy Uniwersytecie Medycznym im. Karola Marcinkowskiego w Poznaniu/ Bioethics Committee of Poznań University of Medical Sciences/. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MW and WP contributed to conception and design of the study. HK, PN, OR, and JM organized the database. OR performed the statistical analysis. HK wrote the first draft of the manuscript. HK, OR, MW, and WP wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.772255/full#supplementary-material

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CASE REPORT
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Complete Response to Nivolumab in Recurrent/Metastatic HPV-Positive Head and Neck Squamous Cell Carcinoma Patient After Progressive Multifocal Leukoencephalopathy: A Case Report

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In an immune-competent context nivolumab showed long-term benefit in overall survival in recurrent/metastatic head and neck squamous cell carcinoma (HNSCC); however, in special cancer population such as these patients with immunodeficiency and viral infections, data on checkpoint inhibitors (ICI) activity are scant. Herein, we report a patient with a Human papilloma virus (HPV)-related oropharyngeal cancer (OPC) and CD4 lymphocytopenia. After a first-line treatment complete remission, the patient experienced Human Polyomavirus (JCV) infection in the brain. Consequently, to the recovery from progressive multifocal leukoencephalopathy (PML) the patient metastasized and was enrolled in a single-arm trial with nivolumab (EudraCT number: 2017-000562-30). A complete and durable response (more 3 years) was observed after 10 nivolumab injections Q2wks, interrupted for persistent drug related G2 diarrhea and a syndrome of inappropriate antidiuretic hormone secretion. We describe the circulating immune profile (before-, during-, and after nivolumab), consistent with the clinical history. Moreover, during nivolumab treatment, brain MRI evidenced the presence of small punctuate areas of contrast enhancement, reflecting a mild immune response in perivascular spaces. By cytofluorimetry, we observed that during JCV infection the CD4/CD8 ratio of the patient was under the normal values. After JCV infection recovery

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and before nivolumab treatment, CD4/CD8 ratio reached the normality threshold, even if the $CD4^+$ T cell count remained largely under the normal values. During ICI, gene expression xCell analyses of circulating immune cells of the patient, showed a progressive normalization of the total immune profile, with significant boost in $CD4^+$ and $CD8^+$ T cells and a reduction in NK T, comparable to the circulating immune profile of reference tumor-free HNSCC patients. The present case supports the activity of ICI in a population of special cancer patients; whether JCV and HPV infections (alone or together) might have a possible role as immune booster(s), require further investigations.

Keywords: immunotherapy, HNSCC, oropharynx, HPV, case report, PML

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the eighth most frequent cancer in the world (1). For patients with recurrent/metastatic (R/M) HNSCC the overall survival has been recently improved due to available new treatment options, especially immune checkpoint inhibitors (ICI, i.e., nivolumab, pembrolizumab). Nivolumab is a human immunoglobulin G4 (IgG4) antibody targeting programmed death-1 (PD-1), an inhibitory receptor on activated T cells. This antibody blocks the interaction with PD-1 receptor and its ligands, PD-L1 and PD-L2, leading to the inhibition of the patient immune response against cancer cells. Nivolumab was approved by the FDA for platinum-refractory R/M HNSCC patients and, as reported in the randomized trial CheckMate-141, showed benefit as compared to standard of care and, once response is obtained, it may be prolonged (2). However, despite those encouraging results, in this set of patients response is still limited (about 15%), and to our knowledge predictive biomarkers, such as PD-L1 expression, are still under investigation (3). At our knowledge, data on the activity of nivolumab in challenging populations (such as immunocompromised patients or those with HIV and viral hepatitis infections) are scant, mainly due to their under representation in clinical trials (4, 5). The present unique case report represents a remarkable portrait of peripheral blood immune profile before, during, and post nivolumab treatment in a platinum-refractory R/M HNSCC patient, with two concomitant viral infections, experiencing a long-term response.

CASE PRESENTATION

A 62-year-old man, an active smoker (>10 packs/year), was referred to our center (IRCCS Istituto Nazionale dei Tumori, Milan, Italy) in March 2017 due to a large lesion of the right infratemporal fossa (55 × 30 × 52 mm). His medical history was unremarkable apart from previous episodes of paroxysmal atrial fibrillation. A graphical representation of the case history is shown in **Figure 1**. Contrast head and neck magnetic resonance imaging (MRI) revealed a large mass with infiltration of the medial pterigoid muscles and the deep parotid lobe, until the lateral nasopharyngeal wall; posterior, the lesion infiltrated the prevertebral muscles, enclosing the right internal carotid together with multiple enlarged nodes in the ipsilateral neck (**Figure 2A**). Fine needle aspiration biopsy of the largest neck node (25 mm) demonstrated a poorly differentiated carcinoma, p16 immuno-histochemistry (IHC) positive in formalin-fixed, paraffin-embedded (FFPE) sections (6) and HPV viral infection confirmed by *in situ* hybridization (ISH). The patient was classified as cT4 N1 p16 positive, stage III according to the AJCC VIII classification. IHC analysis for PD-L1 expression was performed using IHC 22C3 pharmDx kit (Dako, Carpinteria, CA) on FFPE tissue. PD-L1 expression was evaluated both in tumor cells and in inflammatory cells and a combined proportion score (CPS) was determined and tumor infiltrating lymphocytes (TILs) were assessed on hematoxilin & eosin (H&E) slides (7). The analysis showed high PD-L1 expression both in tumor cells (40%) and infiltrating cells (20%) with a CPS = 55. In addition, we observed an enrichment (80%) of TILs. No residual FFPE tissue was available for additional deepen analysis.

FIRST-LINE ONCOLOGIC TREATMENT AND NEUROLOGICAL HISTORY

From June to August 2017 the patient received intensity-modulated radiation therapy (IMRT, 69.96 Gy in 33 fractions) concomitant to 2 courses of carboplatin (AUC 6), due to the previous paroxysmal atrial fibrillation, serum creatinine value at the upper level and tumor related weight loss. The patient obtained a complete clinical remission at the end of the treatment, despite an expected CRT myelotoxicity (**Figure 2B**). In November 2017, due to a subacute progressive gait ataxia and aphasia, a contrast brain head and neck MRI was performed (data not shown). The image confirmed the complete oncologic remission and in addition showed multiple T2 hyperintense alterations in the supra- and infratentorial white matter, not correlated with the oncological clinical history. From November till December 2017 the patient was treated with steroids (oral cortisone acetate; 62.5 mg daily), but no improvements of neurological symptoms were observed, and the patient was hospitalized at the Fondazione Istituto Neurologico Mondino (Pavia, Italy). Neurological examination revealed moderate cognitive impairment, severe mixed aphasia, mild right

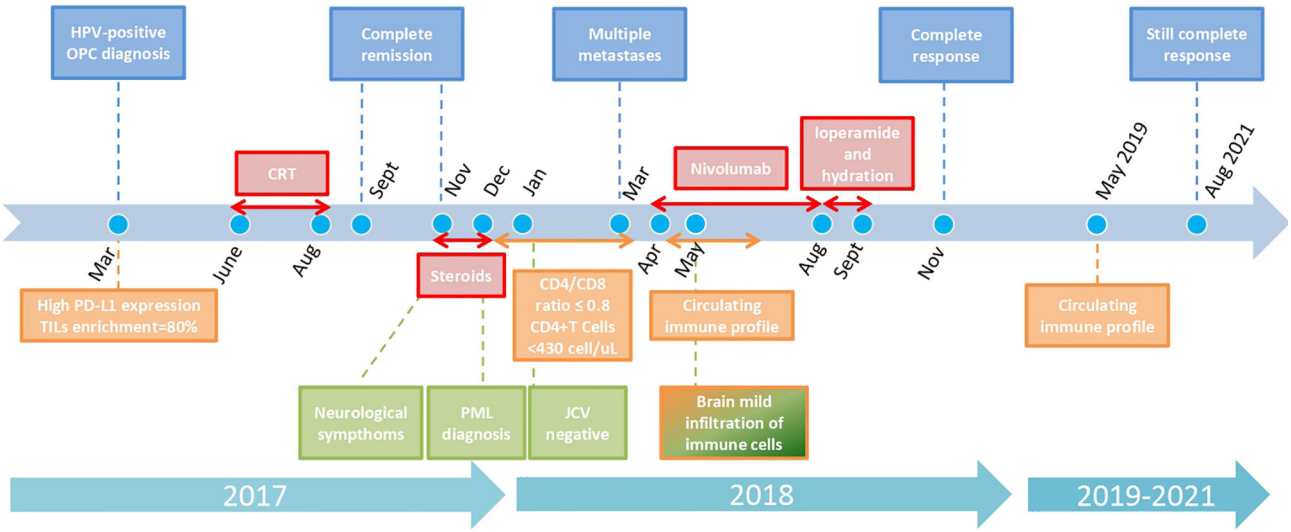

FIGURE 1 | Graphical timeline of case report with key oncologic, neurologic, and immunologic analyses and treatments. Color code for boxes: blue: oncologic history; red: treatments; light green: neurologic history; orange: immunologic analyses results. Every arrow indicates the duration of each treatment/immunologic situation. Dots represent specific key months. The scale is not strictly proportioned to dates.

hemiparesis, and cerebellar ataxia. The patient needed support for most daily life activities (modified Rankin scale—mRS—equal to 3) (8). Cerebrospinal fluid (CSF) analysis showed no inflammatory findings and no oligoclonal banding; Human Polyomavirus JC (JCV) DNA was isolated from 150 ml of CSF, using the commercial kit Nuclesopin RNA virus (Macherey Nagel, Germany) and Real Time PCR, targeting the JCV Large- Antigen gene (9). Active JCV replication (4,700 copies/ml) was observed, and a brain MRI performed in December (**Figures 3A–D**), identified a large lesion in the left fronto-parietal white matter, characterized by hyperintensity on T2/FLAIR images, mild mass effect, and no contrast enhancement after gadolinium administration; b1000 images showed small areas of increased signal in a star-like («milky way») distribution pattern, though with incomplete correspondence hyposignal in the ADC map, which is a typical finding in "definite progressive multifocal leukoencephalopathy (PML)", reflecting diffusion restriction due to active demyelination. Steroid was withdrawn and over the following weeks, the patient experienced a gradual neurological improvement. Since January 2018, JCV viral load became undetectable in the CSF. In March 2018, aphasia and cognitive deficits were substantially improved and he was able to walk independently (mRS 2). Blood tests of lymphoid components, through cytofluorimetric analysis, were performed from December 2017 to March 2018. In December, during steroid treatment, CD4/CD8 ratio was inverted (CD8 >CD4; CD4/CD8 = 0.6, normal threshold >0.8), and the number circulating $CD4^+$ T cells were below the normal threshold (between 430 and 1,600 cells/μl) with an impaired count of 73 $CD4^+$ cells/μl. In January 2018, with the withdrawal of steroid, a slight improvement in $CD4^+$ T cell count was observed (161 cells/μl) and CD4/CD8 ratio was restored (CD4 >CD8, CD4/CD8 = 1.4). In March 2018, CD4/CD8 ratio was in the normal threshold (CD4/CD8 = 08), and $CD4^+$ T cell count was slightly increasing 165 cell/μl. However, $CD4^+$ T cells remained largely under the normal threshold.

MULTIPLE DISTANT METASTASIS AND SECOND-LINE TREATMENT

In March 2018, multiple distant metastases in skin, soft tissues, and bilateral lungs have been found at the follow-up 18FDG-PET scan (**Figures 2C, D**). In April 2018, the patient was enrolled in the study "A Single-Arm, Open-Label, Multicenter, Trial with Nivolumab in Subjects with Recurrent or Metastatic Platinum-refractory Squamous Cell Carcinoma of the Head and Neck" (EudraCT number: 2017-000562-30). During the following six months, the patient received 10 well-tolerated nivolumab infusions, each one of 240 mg every two weeks. A brain MRI, performed after the 4th nivolumab infusion (**Figures 3E–H**), showed a reduction in the extension of the left fronto-parietal lesion that no longer displayed mass effect, as evident on FLAIR images; b1000 images now showed an extensive area of hyposignal with correspondent hypersignal on ADC, reflecting extensive white matter destructuration without signs of ongoing active demyelination; areas of slight increased b1000 signal were still evident more anteriorly. T1 images after gadolinium injection showed the appearance of small punctuate areas of contrast enhancement, reflecting a mild immune response in perivascular spaces. Only a mild aphasia and apraxia without further deficits were present and JC viral load in the CSF continued

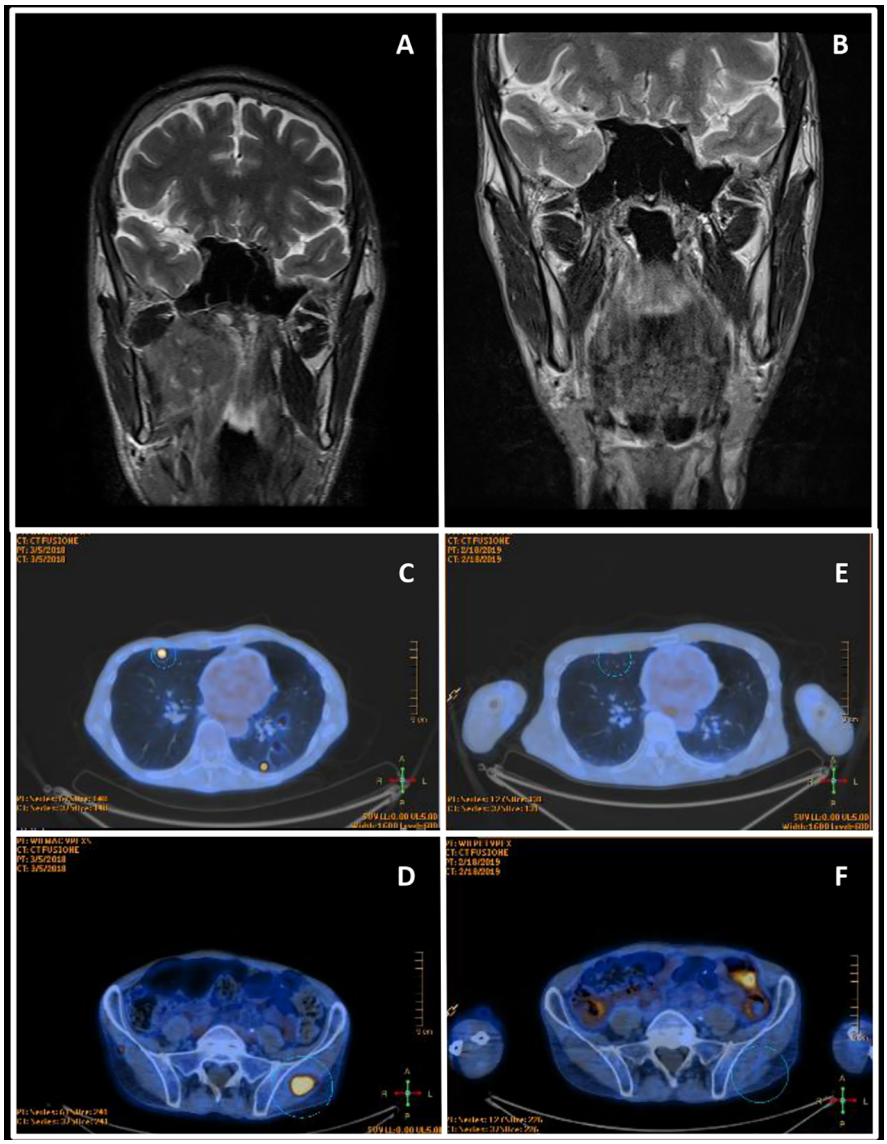

FIGURE 2 | Radiological assessments. **(A)** March 2017 Head and neck contrast-enhanced magnetic resonance imaging—before the primary treatment. **(B)** November 2017 Head and neck contrast-enhanced magnetic resonance imaging of complete remission after chemoradiation. **(C, D)** March 2018 Whole body FDG PET—at diagnosis of metastatic disease. **(E, F)** November 2018 Whole body FDG PET of complete response after nivolumab.

to be undetectable. Blood tests, through cytofluorimetric analysis, at the 5th cycle of nivolumab detected a CD4/CD8 ratio in the normal threshold (CD4/CD8 = 08), while $CD4^+$ T cell count remained largely under the normal values (160 cells/μl). In June 2018 a partial response according to the RECIST 1.1 criteria (5) was evident at the CT scan. In September 2018, nivolumab was withdrawn after 10 infusions, due to a G2 diarrhea (probably drug-related), persistent despite symptomatic treatment, and a syndrome of inappropriate antidiuretic hormone secretion (SIADH). Diagnosis of immune-mediated colitis was not endoscopically confirmed. Side effects were managed with loperamide and hydration, without using steroid or immunosuppressive therapy because of the risk of JC reactivation. In January 2019 a contrast brain MRI (**Figures 3I–L**) confirmed a substantial stability of imaging findings, disappearing of diffusivity abnormalities, but further evolution of cortical atrophy, even more evident at later follow-up evaluations. T1 images after gadolinium showed the disappearance of the small punctuate areas of enhancement that were previously evident during nivolumab treatment. In November 2019, a complete remission was confirmed by a CT scan total body (**Figures 2E, F**). At date, October 2021, the patient is still in complete remission for the HPV-related OPC and the JC infection.

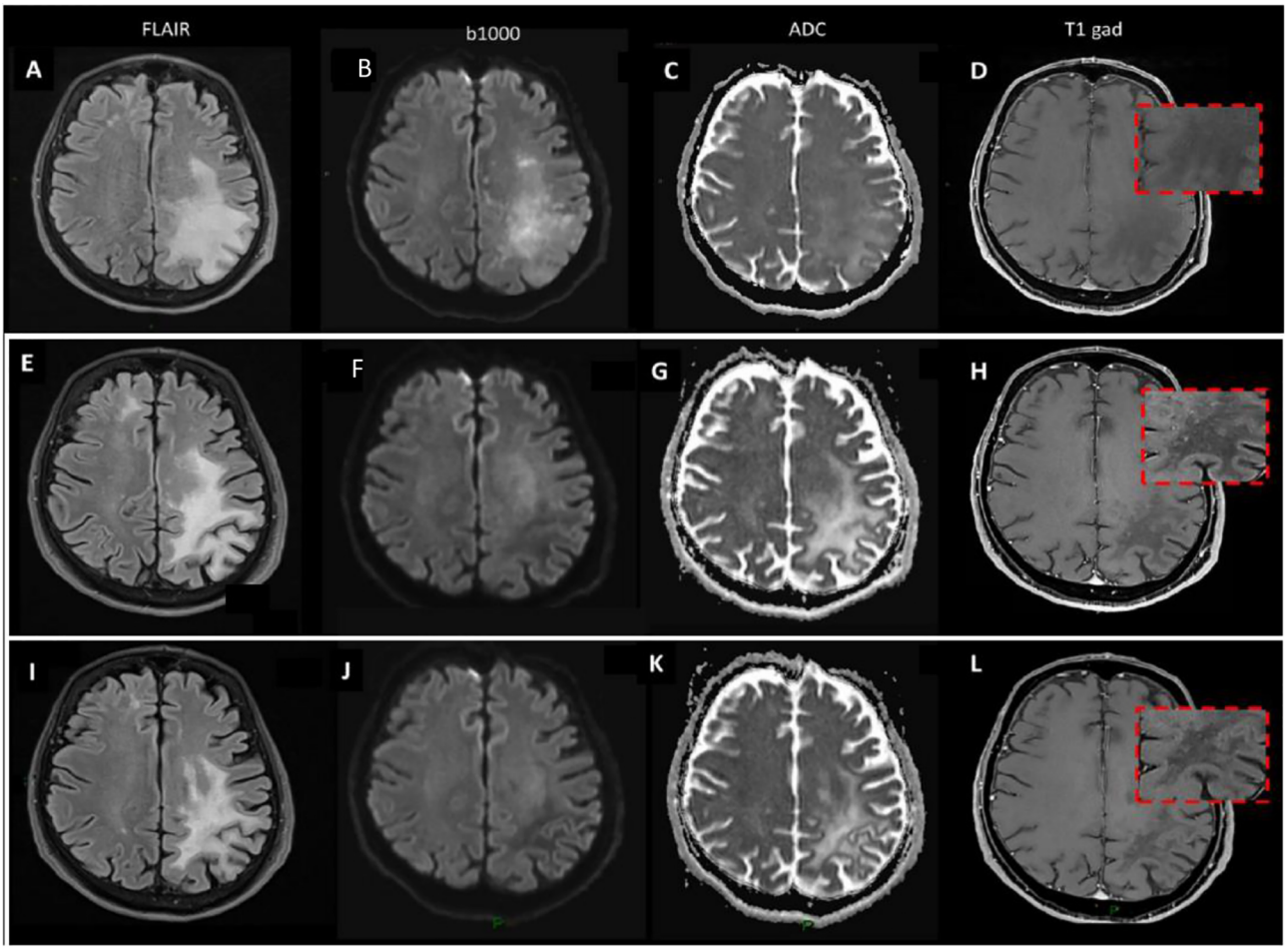

FIGURE 3 | Brain MRI. **(A–D)** December 2017 brain MRI at PML diagnosis. **(E–H)** May 2018 brain MRI during ICI treatment. **(I–L)** January 2019 brain MRI after ICI treatment.

CIRCULATING IMMUNE-PROFILE RESULTS

Recognizing the importance of profiling the immunological characteristics of the patient, blood samples collected before, during and after the single agent nivolumab treatment were employed to investigate, through a de-convolution gene expression method, the immune cells populations present in the peripheral blood. As reference, blood samples were also collected from 5 different HNSCC patients tumor-free after radical treatment. The reference patients were in the same age range and of the same gender (but one) of our patient, while they are heterogeneous in smoking history (3 heavy ex-smokers, 2 never smokers), site of the primary disease (two were p16 positive OPCs, 3 oral cavity cancer). Blood collection was made after signed informed consent from each patient. To perform the analysis, RNA was extracted from frozen blood buffycoat using RNeasy Lipid Tissue Mini Kit (Qiagen). Quality/quantity RNA had been assessed by 2200TapeStation system (Agilent), and Qubit 2.0 Fluorimetric Assay (Thermo Fisher Scientific), respectively. Gene expression experiments were performed using GeneChip WT Pico standard protocols (Affymetrix, ThermoFisher) on human Clariom-S chips. Probes were hybridized on GeneChip, that after washing and staining, through the Fluidics Station, were scanned with Affymetrix Gene Chip Scanner 3000 7G. Microarray data were compliant to MIAME (Minimum Information about a Microarray Experiment) and two gene expression matrices (5 different temporal samples from case report; 5 samples from reference patients) were deposited on GEO (accession number GSE161785). Bioinformatic analysis were performed on two different expression matrices, by the xCell approach (10).

Lymphoid cells with evident changes during and after nivolumab are reported in **Figure 4** (see **Supplementary Table** for all the xCell results and related statistics). At the beginning of April 2018 ("before-nivolumab"), all the considered lymphoid cells, with exclusion of NKT and $CD8^+$ Tem, exhibited a lower or absent expression compared to controls. In May and June 2018

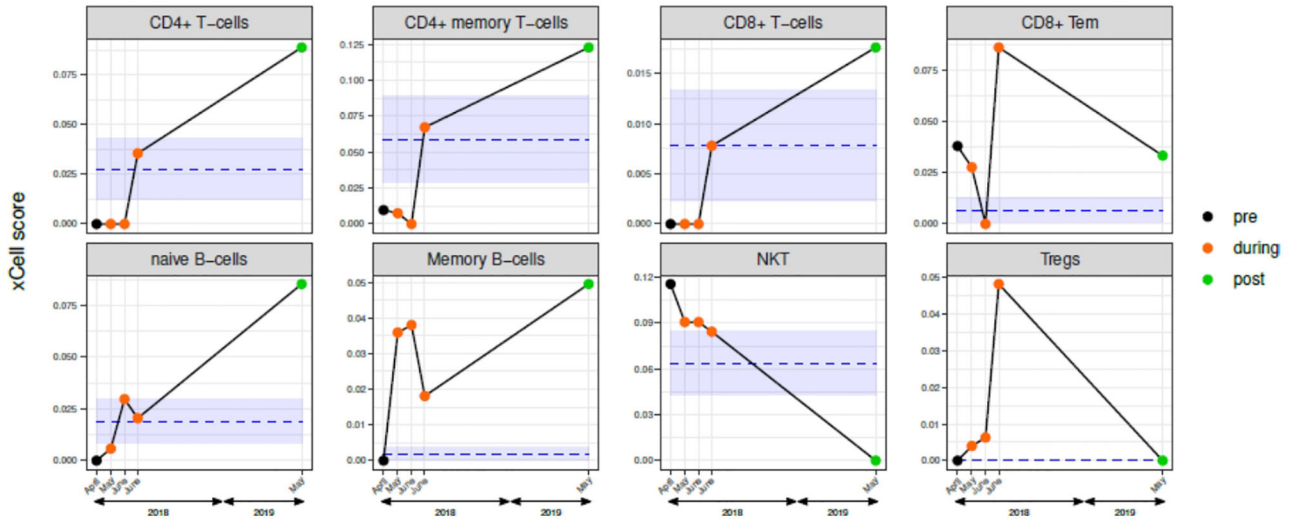

FIGURE 4 | Circulating immune cells characterization before, during, and after nivolumab treatment. Scores of selected lymphoid cells determined by xCell analysis of gene expression data of blood samples from: five samples case report patient (one pre-nivolumab: black dot; three during-nivolumab: orange dots; one post-nivolumab: green dot); mean and standard deviation of the five-reference xCell scores of tumor-free patients are represented by blue lines and light blue area, respectively. See **Table S1** for xCell data for the complete xCell scores ("lymphocytes" and "non-lymphocytes").

in two different blood samples ("during-nivolumab"), no relevant differences were observed for $CD4^+$ T cells, $CD4^+$ memory T cells, $CD8^+$ T cells, naïve B cells, and Tregs, compared to the baseline expression. On the contrary, a decrease for $CD8^+$ Tem and NKT and an increase for memory B cells were recorded. In comparison with the references' range of expression, the major immunological profile changes were recorded in the 3rd sample (7th nivolumab infusion): $CD4^+$ T cells, $CD4^+$ memory T cells, $CD8^+$ T cells, naïve B cells, NKT entered in the range, while $CD8^+$ Tem, memory B cells, and Tregs overtook the reference range. Interestingly, the immunological boost observed during the last sample "during-nivolumab", overtaking the controls, has been maintained one year later in May 2019.

DISCUSSION

We describe here the complete clinical and radiological remission of a metastatic, platinum-refractory, HPV-related OPC with ICI favorable markers (tumor PD-L1 and TILs IHC data) (3) in a patient who, after a partial recovery from CRT myelotoxicity and a complete recovery from a JC viral infection, received nivolumab. To our knowledge this is the first report of a complete durable response after ICI in a cancer patient with two viral infections (HPV in the tumor and JC in the cerebral system). Immunotherapy has been clinically used for the treatment of PML in patients with hematological malignancies, HIV-infection, or primary immune deficiencies, with erratic and conflicting results. In fact, while most patients show a decrease of PD1 expression on T-$CD4^+$ and $CD8^+$ lymphocytes as a result of PD1 blockade, only a proportion of them shows a consensual improvement of PML and even negative effects in transplant-receiver patients (11). In our case, PML was challenging and lately diagnosed since the patient had no personal history suggesting a primary immune deficiency and nivolumab was administrated after a transient immune suppression and PML remission. In the PML patients treated with ICI (12, 13), T cell profile seems the major determinant in eliciting a clinical and radiological response, being patients with higher amounts of terminally exhausted T cells less likely to respond to PD1 blockade (11, 14). Similar observations have been reported in patients receiving ICI for cancer treatment (15) and specifically in HNSCC (16). For these reasons, we decided to analyze the circulating immune profile of the case report patient before, during and after nivolumab, using the gene expression of frozen buffy coat and, as reference, HNSCC patients tumor-free after first-line treatment. The choice was based on the available material according to the trial protocol and on the evidence that gene expression data are comparable to cytofluorimetric data (10). At baseline we observed an immunoprofile consistent with the clinical history of our patient, with low levels of effector T cells ($CD4^+$ and $CD8^+$), and higher levels of $CD8^+$ memory and NKT-cells, in contrast with those reported for HNSCC patients (13), where, in an immunocompetent context, the response to nivolumab at baseline was associated with higher levels of $CD8^+$ T cells and lower levels of PD-1^+ Tregs. Even if the baseline immune profile of our patient could be interpreted as a negative predictor of response, the significant increase in relative levels of total $CD4^+$ T and memory B cells during ICI, maintained after the end of nivolumab, was in agreement with ICI efficacy. In addition, if we consider the particular immune status of this patient and we compare our data with those of PML

patients, the increase of $CD4^+$ T cells during treatment seems consistent with the observation that $CD4^+$ T cell counts increased during ICI in responding PML patients (15). Indeed, the brain MRI during ICI showed the appearance of small punctuate areas of contrast enhancement within PML lesions, not present at the time of PML diagnosis, which spontaneously resolved, after nivolumab discontinuation. These areas, similarly to literature reported cases of ICI treated PML (17), are described as immune cell infiltrates in perivascular spaces and might be due to a collateral nivolumab boosting of a JC-specific cellular immunity. Nonetheless, PML was already evolving favorably in our patient before the start of nivolumab, questioning the contribution of nivolumab in JC virus clearance. Overall, the immune profile changes during treatment and the clinical outcome suggested that the immunotherapy was effective despite a persistent $CD4^+$ lymphocytopenia. Although the immune system impairment, we decided to no deepening into the lymphopenia of our patient since it might be specifically attributed to chemo-radiation treatment and steoroid administration, frequently recorded in HNSCC patients after primary treatment (18).

We should acknowledge some limitations of our study. First, the lack amount of available tumor tissue precluded the comparison between circulating immune cells with tumor immune microenvironment. Second, in adherence to the study protocol, the use of the entire blood samples for RNA extraction precluded a deeper evaluation of the intriguing Tregs expression during treatment, and long-term maintenance of high levels of memory B cells in comparison with samples of references. Third, the comparison with the literature reports (17) is indirect because an absolute counts of immune cells by cytofluorimetry has been reported in the literature, while our immune-profile was inferred from gene expression data. In conclusion, nivolumab induced a durable and complete response in a metastatic HNSCC patient with CD4 lymphocytopenia, experiencing two consecutive viral infections such as HPV and JCV. Our findings support the use of ICI in immune compromised subjects but whether JCV alone or in association with HPV infection could act as immune booster(s) requires further investigations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in GEO repository (ID GSE161785). The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161785.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conception and design: LDL, LDC, and SiC. Financial support: LDC. Provision of study material: LDL and FP. Collection and assembly of data: LDL, EO, SD, GB, AP, and EM. Experimental data generation: MSS and LDC. Data analysis and interpretation: MSS, LDC, AC, SiC, StC, EM, and LFL. Manuscript writing: SiC, LDL, MSS, StC, LDC, and EM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.799453/full#supplementary-material

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Defining a Standard Set of Health Outcomes for Patients With Squamous Cell Carcinoma of the Head and Neck in Spain

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Purpose: A systematic, standardized collection of health outcomes during patient treatment and follow-up, relevant from the perspective of all stakeholders, is a crucial step toward effective and efficient disease management. This project aimed to define a standard set of health outcomes for patients with squamous cell carcinoma of the head and neck (SCCHN).

Methods: The project was led and coordinated by a scientific committee (SC). It comprised: (1) a literature review (to identify variables used during SCCHN management); (2) 1st-SC meeting (to select the variables for presentation during nominal groups-NG); (3) five NG (n=42 experts) and four interviews with patients (to reach consensus on the variables for inclusion); and (4) final-SC meeting (to review the results of NG ensuring consensus on the variables where consensus was not reached).

Results: Experts agreed to include the following variables in the standard set: treatment-related (treatment intent and type, response to treatment, treatment toxicity/complication, treatment completion), degree of health (performance status, patient-reported health status, pain, dysphonia, feeding and speech limitations, body image alteration, tracheotomy), survival (overall and progression-free survival, cause of death), nutritional (weight, nutritional intervention), other variables (smoking status, alcohol consumption,

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patient satisfaction with aftermath care, employment status), and case-mix variables (demographic, tumor-related, clinical and nutritional factors).

Conclusions: This project may pave the way to standardizing the collection of health outcomes in SCCHN and promote the incorporation of patients' perspective in its management. The information provided through the systematic compilation of this standard set may define strategies to achieve high-quality, patient-centered care.

Keywords: head and neck cancer, patient-centered care, outcome measurement, patient-reported outcomes, patient centricity, quality of life

1 INTRODUCTION

Head and neck cancer includes a group of neoplasms of various anatomical sites that differ in terms of etiology, diagnostic and treatment approaches (1). It was the seventh most common cancer worldwide in 2020 accounting for 932,000 new cases and 466,500 deaths (2). In Spain, it was estimated that 14,200 new cases of head and neck cancer would be diagnosed in 2020 (3). More than 90% of cases are squamous cell carcinomas of the head and neck (SCCHN) (4) and more than 60% of patients with SCCHN present with stage III or IV disease (5). SCCHN is typically diagnosed in older patients, with smoking and alcohol consumption being two of the main risk factors for its development (1). Moreover, human papilloma virus (HPV) infection has emerged as a new risk factor, especially in oropharyngeal cancer (6).

Treatment for SCCHN is complex and requires a multidisciplinary approach since it differs according to the stage of the disease, anatomical site, and surgical accessibility. It may require intricate surgery, radiation, chemotherapy, and/or targeted therapy, and/or immunotherapy (7, 8). Some of these treatment options involve changes to critical structures for speaking, eating and breathing, which can lead to functionality problems. Therefore, as a result of treatment, patients with SCCHN face long-term challenges beyond surveillance for recurrent or secondary cancer, including adapting to disfigurement, managing dysphagia and developing alternative speech (9, 10).

In addition to curative intention, structural and functional preservation, amelioration of morbidities when feasible, and long-term maintenance of health-related quality of life (HRQoL) are the principal treatment objectives. Therefore, treatment selection based on a multidisciplinary tumor board decision is essential (11, 12). There is growing evidence supporting the routine collection of patient-reported outcomes (PROs) to enable improved, patient-centered care. The systematic collection of PROs in oncology has a positive effect on patient-physician communication, improves the monitoring of disease progression and response to therapy, helps identify unrecognized problems (physical, emotional and/or social problems), contributes to detecting adverse effects of treatment, and enhances patients' experience and satisfaction (13). Despite the collection of PROs being considered the cornerstone for achieving the best results and preserving patients' HRQoL, their systematic collection using standardized and validated instruments is mostly limited to clinical research environment, with scarce use in clinical practice.

To move toward an effective and efficient patient-centered system, a holistic approach is required, integrating evidence from clinical outcomes and PROs. Experience gained from other fields shows that the systematic and standardized collection of outcomes is the *sine qua non* to improve the quality of any process (14). During the last few years, pioneer initiatives such as the one performed by the International Consortium for Health Outcomes Measurements (ICHOM) (15) have focused on the development of standard sets of health outcomes for various diseases, among which SCCHN is not included. The long-term goal of these initiatives is to promote consistency in data collection between different institutions within the same country or among different countries. A systematic, standardized compilation of health outcomes, relevant from the perspective of all stakeholders, during patient treatment and follow-up, is a key step toward effective and efficient disease management. This project aimed to define a standard set of health outcomes and the most appropriate instruments to measure them for managing patients diagnosed with SCCHN. This is the first step to ensure to standardize the collection of health outcomes in SCCHN.

2 MATERIALS AND METHODS

The project comprised four phases: (1) a literature review; (2) first scientific committee meeting; (3) five nominal groups (described in greater detail below) and four semi-structured interviews with patients, and; (4) final scientific committee meeting (**Figure 1**). The scientific committee and nominal group meetings were conducted between June 2019 and December 2020. The project was led and coordinated by a scientific committee consisting of healthcare professionals who are experts in the management of SCCHN, and/or have experience in implementing strategies to standardize health outcomes (three specialists in medical oncology [VA, RM, AR], one specialist in radiation oncology [JG], one specialist in otolaryngology [AS], one specialist in oral and maxillofacial surgery [FM], one specialist in quality and innovation [DC], one hospital pharmacist [GC]) and one representative of a

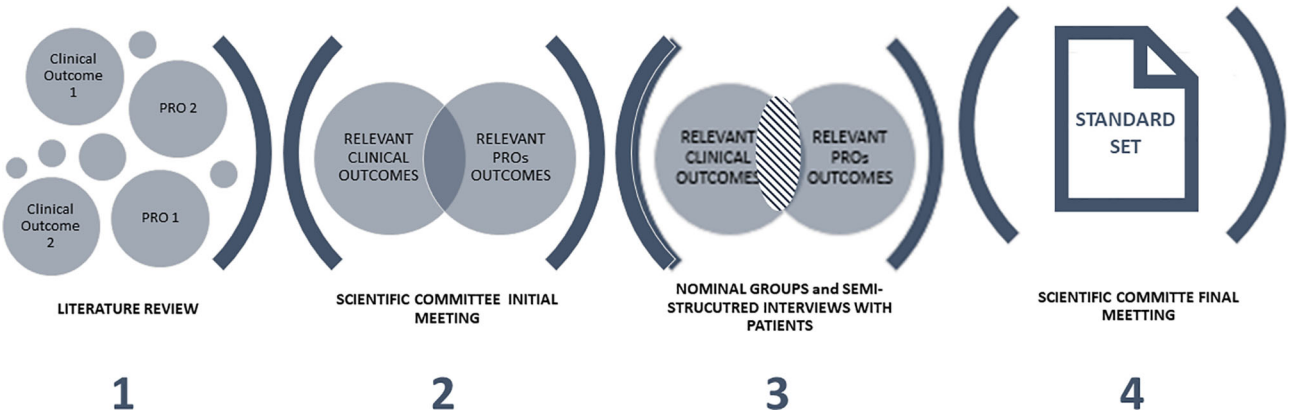

FIGURE 1 | Project phases.

Spanish patient advocacy group (*Grupo Español de Pacientes con Cancer*, GEPAC).

2.1 Literature Review

To identify health outcomes [clinical and patient-reported outcomes (PROs)], instruments, and frequency of measurement to be used during SCCHN patient follow-up, a systematic literature review according to the Cochrane Handbook for Systematic Reviews of Interventions (16) (**Supplementary Table S1**), was carried out in Medline/PubMed. Clinical trials or systematic reviews that include clinical trials in SCCHN, published in English and/or Spanish between 01/01/2016 and 03/31/2019 were reviewed.

2.2 First Scientific Committee Meeting

The first meeting with the members of the scientific committee aimed to present the project, define the target population and, based on the results of the literature review, select the health outcomes for presenting during the nominal groups.

During the discussion group, the scientific committee screened health outcomes (variable/instrument/frequency of measurement) identified in the literature review and selected them according to their relevance for patient follow-up and availability in the Spanish setting. Moreover, the scientific committee proposed new health outcomes not previously identified in the literature review, but relevant from their perspective.

2.3 Nominal Group Meetings and Semi-Structured Interviews With Patient Representatives

Five nominal multidisciplinary groups were conducted to reach a consensus on the health outcomes for inclusion in the standard set. Nominal group meetings took place either face-to-face (n=4 meetings) or online (n=1 meeting), depending on participants' availability to meet.

A nominal group is a qualitative methodology that allows reaching a consensus and ensuring balanced participation among group members, giving them equal opportunities to share their opinions (17). Following the methodological recommendation (18), nominal groups involved five main steps: 1) Introduction and explanation: welcome and description of the purpose and procedure of the meeting; 2) Silent generation of ideas: each participant individually (without consulting or discussing with others) evaluated the health outcomes proposed; 3) Sharing ideas: separately, participants shared the health outcomes they had selected; 4) Group discussion: participants could seek a verbal explanation or further details about any of the health outcomes that other participants had proposed; 5) Voting and ranking: during this phase, participants were asked to prioritize the health outcomes proposed. Health outcomes were included if ≥ 75% of participants agreed on their inclusion. The five nominal groups worked on the same standard set (based on scientific committee proposal). An individual consensus on specific standard set was reached in each of the nominal group.

The participation in each nominal group was limited to a maximum of 12 experts. Nominal groups consisted of experts from different geographic areas of Spain, including medical oncologists, radiation oncologists, otolaryngologists, oral and maxillofacial surgeons, a phoniatrician, primary care specialists, hospital pharmacists, hospital managers, psycho-oncologists, nutritionists, speech therapists, and dentists. Members of the nominal groups were identified by the scientific committee, in collaboration with the study coordinator. They were selected based on their experience in SCCHN management, PRO measurement, implementing strategies to standardize health outcomes, as well as their availability and interest in the project.

To gain patients' perspectives on the impact of the disease and its treatment on their day-to-day lives, semi-structured telephone interviews (**Supplementary Table S2**) were conducted with four patient representatives.

2.4 Second Scientific Committee Meeting

The main objective of the last meeting of the scientific committee was to define the final standard set for SCCHN. For this purpose, the agreed health outcomes (clinical and PROs) in each nominal

group were presented to the scientific committee. The scientific committee reviewed the individual results of the five nominal groups, ensuring consensus on the health outcomes for which no agreement was reached among the nominal groups. Therefore, if a health outcome did not reach the consensus of inclusion in the five nominal groups, the scientific committee members assessed its inclusion or exclusion from the final standard set. The consensus was reached if ≥ 75% of the members of the scientific committee agreed on the inclusion/exclusion of the health outcome.

Additionally, results from semi-structured interviews with patients were also presented to confirm that the most relevant health outcomes from the patients' perspective were included in the standard set.

Based on the meeting results, the health outcomes for inclusion in the standard set for SCCHN were defined.

3 RESULTS

3.1 Literature Review

The database search yielded 66 references of which 30 were excluded as their title and abstract contained detailed reviews that did not report health outcomes for patient follow-up. The remaining 36 publications were assessed for eligibility. All of them were selected to be reviewed for qualitative synthesis and identification of health outcomes, measuring instrument, and frequency (**Figure 2**).

A total of 47 health outcomes were identified in the literature review. They were categorized into case-mix variables (baseline factors that may affect the health outcomes but cannot be controlled as part of the management of the condition and enable patient characterization) (n=27) and 20 outcomes variables (variables for patient follow-up) (n=20) (**Supplementary Table S3**).

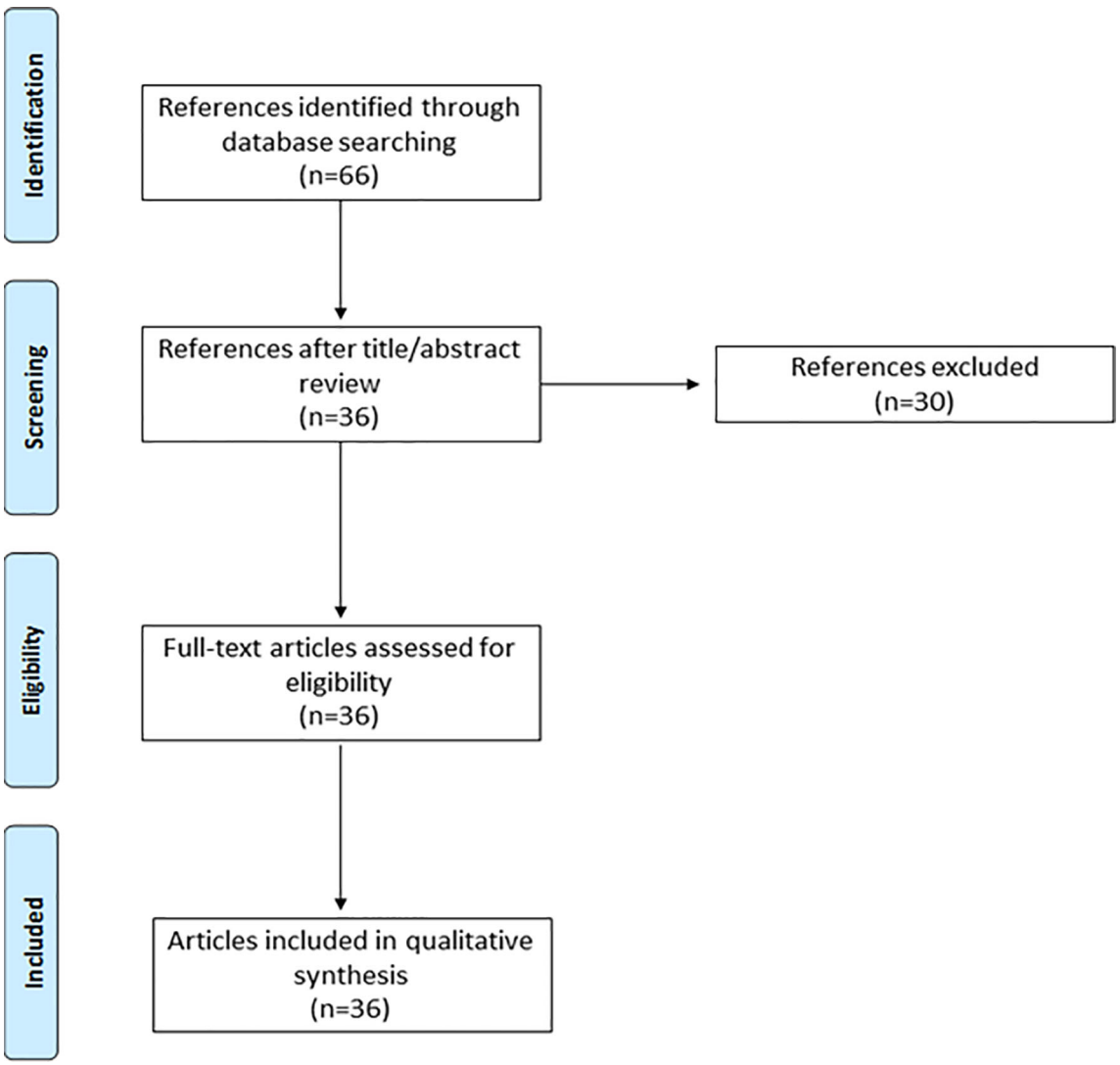

FIGURE 2 | PRISMA flow diagram.

3.2 First Scientific Committee Meeting

The scientific committee considered 18 out of 27 case-mix and 11 out of 20 outcomes variables previously identified in the literature as being relevant. Moreover, two additional case-mix and nine outcomes variables were proposed. Thus, 20 case-mix and 20 outcomes variables were selected by the scientific committee for presentation and evaluation during the nominal groups (**Supplementary Table S4**).

The target population of the standard set was also defined. The scientific committee agreed to include all patients with newly-diagnosed SCCHN originating from the oral cavity, oropharynx, hypopharynx and larynx. Head and neck cancers originating from salivary glands, nasopharynx, paranasal sinuses and nasal cavity, and those with a histological type other than squamous, have special characteristics and are subject to specific recommendations and were therefore not included in the target population of this standard set.

3.3 Nominal Group Meetings

A total of 42 experts on SCCHN from different specialties (n=12 medical oncologists, n=4 radiation oncologists, n=6 otolaryngologists, n=2 oral and maxillofacial surgeons, n=1 phoniatrician, n=1 primary care specialist, n=10 hospital pharmacists, n=1 hospital manager, n=2 psycho-oncologists, n=1 nutritionist, n=1 speech therapist, and n=1 dentist) and geographical areas of Spain participated in four nominal group meetings.

The experts agreed to include twelve case-mix and thirteen outcomes variables proposed by the scientific committee in the standard set. Additionally, thirteen new case-mix and thirteen outcomes variables were proposed during the nominal groups (**Supplementary Table S5**).

3.4 Semi-Structured Interviews With Patients

Four patient representatives participated in the semi-structured interviews (100% men, age range: 54-82 years).

Patients described the impact of the disease and its treatment on HRQoL, mainly due to disease aftermath. The most common symptoms and side effects reported by patients included dry mouth, oral pain, fatigue and loss of taste and smell. Moreover, several dysfunctions such as speech or voice and swallowing problems were also pointed out.

The disease also harmed patients' working lives, as most of the patients who were active at the time of diagnosis were unable to return to work.

3.5 Second Scientific Committee Meeting

Based on the consensus reached among nominal groups, the scientific committee assessed the inclusion or exclusion of the new health outcomes proposed and those for which the nominal groups did not reach a consensus.

3.5.1 Case-Mix Variables

Case-mix variables are defined as factors that may affect the health outcomes but cannot be controlled as part of the management of the condition. The experts agree to collect at baseline the main sociodemographic factors (age, gender, and social/familiar support), tumor-related factors (tumor localization and sublocalization, cancer staging based on TNM status, and date of diagnosis), clinical factors (alcohol consumption; smoking status, performance status, comorbidities, global patient health status, pain, dysphagia, dysphonia, p16 expression, PD-L1 expression, fragility, and referral to dentistry), and nutritional factors (unintentional weight loss, and dental problem) at the time of diagnosis, before initiating treatment (baseline visits) (**Table 1**).

Social/familial support was defined as a proxy for predicting whether the patient would be able to cope with the disease and complete treatment from the physician's perspective (yes/no).

To standardize the assessment of alcohol consumption, a consensus was reached to classify patients as consumers (regular or occasional) or non-consumers from the patient's perspective. Similarly, to collect smoking status at diagnosis in a standardized way, the experts agreed to report if the patient was a never-smoker, an ex-smoker (defined as a patient who has stopped at least one year before diagnosis), or a current smoker. For ex-smoker and current smoker patients, it was agreed to record whether the patient's pack-year index (calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked) was $<$ or ≥ 10.

To systematically report patients' comorbidities, a list of diseases that can influence treatment and/or clinical practice was developed to select patient comorbidities. The list includes: vasculopathy, renal failure, liver failure, anemia, neuropathy, deafness, diagnosed mental illness, other primary cancer, diabetes, COPD, heart disease, and autoimmune disease.

The experts agreed to use validated questionnaires to assess and collect some of these case-mix variables. To describe patients' performance status, experts agreed on the use of the Eastern Cooperative Oncology Group (ECOG) scale, which assesses patients' level of functioning in terms of self-care, carrying out daily activities, and physical ability (19). A generic HRQoL questionnaire (EQ-5D) (20, 21) and head and neck cancer-specific HRQoL questionnaire [EORTC QL-Q H&N43 (22)] were proposed to assess patients' global health status and the impact of the disease on their social, working and personal function. The use of EORTC QL-Q H&N43 also allows gathering information about patients' perspective of pain, dysphagia, and dysphonia. And finally, to evaluate patients' fragility, the experts agreed to complete the G8 questionnaire (23) in patients over 70 years of age.

3.5.2 Outcomes Variables

Outcomes variables establish the evolution of patients' health status and determine the response (success) in managing the medical condition (**Table 2**). They are collected during patient follow-up.

3.5.2.1 Treatment-Related Variables

It was agreed to collect, prior to initiating treatment, data on treatment intention and type. For patients that receive curative treatment, the experts reached a consensus on the inclusion of

TABLE 1 | Spanish standard set of patient-centered outcomes in SCCHN.

Patient profile	Variable	Supporting information	Measurement instrument	Timing	Data sources
Sociodemographic factors					
All patients	Age		Date of birth	Baseline (before treatment begins)	Clinical report
	Gender		F: female; M: male		Clinical report
	Family support	Assessment of patient's environment/support as a proxy for predicting whether he/she will be able to cope with the disease and complete treatment	(1) Yes; (2) No		Physician-reported
Baseline tumor factors					
All patients	TNM status		TNM scale	Baseline (before treatment begins)/ after pathological anatomy results (if available)	Clinical report
	Tumor localization and sub localization		NA		Clinical report
	Date of diagnosis		NA		Clinical report
Baseline clinical factors					
Patients with oropharyngeal cancer	p16 expression		(1) positive; (2) negative		Clinical report
Recurrent metastatic cancer	PD-L1 expression		(1) positive; (2) negative		Clinical report
Patients >70 years	Fragility		G8 questionnaire		Physician-reported
All patients	Alcohol consumption	Alcohol consumption at diagnosis	(1) Consumer (regular or occasional consumer); (2) Non-consumer	Baseline (before treatment begins)	Physician-reported according to patient-notification
	Smoking status	Smoking status at diagnosis	1) Never-smoker; (2) Ex-smoker (stopped >1 year before diagnosis): years + PYI <10 or PYI ≥10; (3) Current smoker: PYI <10 or PYI ≥10		Physician-reported according to patient-notification
	Performance status		ECOG scale		Physician-reported
	Comorbidities		Comorbidities list*		Clinical report
	Patient-reported health status	Global health and impact of the disease on physical, social and emotional function	Tracked *via* generic questionnaire EQ5D and H&N specific questionnaire EORTC QL-Q H&N43		Patient-reported
	Pain		Tracked *via* items 31,32, 34, 63 of EORTC QL-Q H&N43		Patient-reported
	Referral to dentistry		(1) Yes; (2) No		Physician-reported
	Dysphagia	Swallowing problems	Tracked *via* items 35-38 of EORTC QL-Q H&N43		Patient-reported
	Dysphonia		Tracked *via* items 47, of 55-58 EORTC QL-Q H&N43		Patient-reported
Baseline nutritional factors					
All patients	Unintentional weight loss	Unintentional weight loss during the previous 3 months	Yes/No/I don't know	Baseline (before treatment begins)	Patient-reported
	Dental problems	Loss of teeth or dental problems	Tracked *via* items 39, 40 and 73 of EORTC QL-Q H&N43		Clinical report

*PYI, Pack-year index; NA, not applicable; ECOG, Eastern Cooperative Oncology Group; PD-L1, Programmed death-ligand; EQ-5D, EuroQol; EORTC, quality of life core questionnaire; H&N, head and neck cancer; *includes: vasculopathy, renal failure, liver failure, anemia, neuropathy, deafness, diagnosed mental illness, other primary cancer, diabetes, COPD, heart disease, and autoimmune disease.*

Case-mix variables.

treatment response at three months after end of treatment. For patients receiving palliative treatment, it was agreed to assess response to treatment using RECIST criteria every three cycles of treatment.

The experts agreed to report if the patient had developed treatment toxicity or any surgical complication interfering with or modifying the treatment plan. Finally, the experts considered it relevant to indicate if the patient had completed

TABLE 2 | Spanish standard set of patient-centered outcomes in SCCHN.

Patient profile	Measure	Supporting information	Measurement instrument	Timing	Data sources
Treatment variables					
All patients	Treatment intent		(1) curative; (2) palliative	Baseline (before treatment begins)	Physician-reported
	Type of treatment		(1) Surgery; (2) Radiotherapy; (3) Chemotherapy: (4) Immunotherapy; (5) Targeted therapy; (6) Supportive therapy	Baseline (before treatment begins)	Physician-reported
	Response to curative treatment		(1) Disease-free; (2) Persists; (3) Progress	At 3 months after treatment ends	Clinical report
	Response to palliative treatment	Using RECIST criteria	(1) Complete response; (2) Partial response; (3) Progressive disease; (4) Stable disease	Every 3 cycles of treatment	Clinical report
	Treatment toxicity or surgical complication	Development of treatment toxicity or surgical complications that have interfered with or modified treatment plan	(1) Yes; (2) No	During treatment or surgery	Physician-reported (according to clinical report or patient's perspective)
	Treatment plan completed		(1) Yes; (2) No; due to lack of efficiency; (3) No, due to toxicity; (4) No, due to patient's death; (5) No, due to intermittent cause	At treatment end	Physician-reported
Degree of health					
All patients	Performance status		ECOG scale	1st year: every 3 m/2nd-3rd years: every 6 m/later: every year	Clinical report
	Patient-reported health status	Global health status, physical and emotional function	Tracked *via* generic questionnaire EQ5D and SCCHN specific questionnaire EORTC QL-Q H&N43	1st year: every 6 m/later: every year	Patient-reported
	Pain		Tracked *via* items 31-34, 63 of EORTC QL-Q H&N43		Patient-reported
	Dysphonia		Tracked *via* items 47, of 55-58 of EORTC QL-Q H&N43		Patient-reported
	Feeding limitations	Includes: dysphagia, dental problems, xerostomia, taste/smell alteration, chewing/eating problems	Tracked *via* items 35-45, 51-54, 73 of EORTC QL-Q H&N43		Patient-reported
	Oral communication limitations	Includes hoarseness and problems talking	Tracked *via* items 47, 55-58 EORTC QL-Q H&N43		Patient-reported
	Body image alteration	Include body image and sexual limitation	Tracked *via* items 48-50, 59-61 of EORTC QL-Q H&N43		Patient-reported
	A requirement for permanent tracheotomy		(1) Yes; (2) No	When tracheotomy is required	Clinical report
Survival					
All patients	Overall survival		Date of death	NA	Administrative data (death registry)
	Progression-free survival		NA	1st year: every 3 m/2nd-3rd years: every 6 m/later: every year	
	Cause of death	Tumor/treatment-related or not	NA	NA	Administrative data (death registry)
Nutritional variables					
All patients	Weight		NA	1st year: every 3 m/2nd-3rd years: every 6 m/later: every year	Clinical report
	Nutritional intervention	Nutritional intervention required during treatment or follow-up	(1) Yes, oral supplementation; (2) Yes, tube enteral nutrition tube; (3) yes, enteral nutrition by ostomy; (4) Not required	When nutritional intervention is required	Clinical report
Others					
All patients	Smoking status	Reported if patient still smokes:	(1) Yes; (2) No; (3) Patient did not smoke prior to diagnosis	1st year: every 3 m/2nd-3rd years: every 6 m/later: every year	Physician-reported according to patient-notification
	Alcohol consumption	Reported if patient still consumes alcohol	(1) Yes; (2) No; 3) Patient did not drink alcohol prior to diagnosis		

(Continued)

TABLE 2 | Continued

Patient profile	Measure	Supporting information	Measurement instrument	Timing	Data sources
				1st year: every 3 m/2nd-3rd years: every 6 m/later: every year	Physician-reported according to patient notification
	Patient satisfaction with aftermath care		(1) Satisfied;(2) Not satisfied; (3) Patient does not have an aftermath	After hospital discharge or 5 years after treatment ends	Patient-reported
	Employment status	Record whether the patient has been able to return to their previous job in the same conditions	(1) Yes;(2) No; (3) Patient did not work prior to diagnosis	After oncology department discharge or 5 years after treatment ends	Patient-reported

NA, not applicable; ECOG, Eastern Cooperative Oncology Group; EQ-5D: EuroQol; EORTC, quality of life core questionnaire; H&N: head and neck cancer. Outcomes variables.

the treatment plan and, when applicable, record the reason for not doing so.

3.5.2.2 Degree of Health

SCCHN has a negative impact on patients' performance and health status. Therefore, it was agreed to collect both sets of variables during patient follow-up. As previously indicated, the ECOG scale (19) was selected to assess patients' performance status, while the EQ-5D (20, 21) and EORTC QL-Q H&N43 (22) were chosen for the assessment of patients' HRQoL. The use of EORTC QL-Q H&N43 annually during patient follow-up allows the evaluation of the impact of the disease on patients' lives, including pain, dysphonia, feeding limitations, oral communication limitations, and body image alterations.

Given the significant impact that it can have on patients, it was also agreed to collect information on whether or not the patient requires a permanent tracheotomy.

3.5.2.3 Survival

Overall survival and progression-free survival were considered key variables for inclusion in the standard set for patient follow-up. Moreover, participants agreed on gathering information regarding cause of death, indicating whether it was tumor- or treatment-related.

3.5.2.4 Nutritional Variables

The disease and its treatment have a negative impact on patients' nutritional status. For this reason, it was agreed to collect patients' weight at each visit, and record whether the patient had required nutritional intervention during treatment or follow-up.

It is important to note that the use of EORTC QL-Q H&N43 allows the physician to assess dysphagia, dental problems, xerostomia, taste/smell alterations, and chewing/eating problems that may impact patients' nutritional status.

3.5.2.5 Others

Alcohol and tobacco consumption are the main risk factors for SCCHN development, and their maintenance during and/or after treatment is related with recurrence, second neoplasms and tobacco/alcohol-related death. Consequently, it was agreed to record whether the patient continued smoking or consuming alcohol after diagnosis.

SCCHN may also affect patients' employment status; therefore, the experts agreed to report whether patients can return to their previous job under similar conditions after discharge from the oncology department or five years after end of treatment.

Most patients with SCCHN reported difficulty in access to aftermath care. Therefore, it was agreed to record whether patients were satisfied with the aftermath care received after being discharged from the oncology department and five years after treatment end.

4 DISCUSSION

A systematic and standardized collection of health outcomes during follow-up of patients with SCCHN is a crucial step toward a more effective and efficient healthcare system. A holistic approach, integrating all stakeholders' perspectives, is necessary to ensure the best quality care. To this end, a standard set that includes relevant health outcomes from the perspective of both patients and healthcare professionals is required. The SCCHN standard set defined herein is an excellent opportunity to promote patient-centered care and optimize SCCHN management.

The SCCHN standard set includes 21 outcomes variables. In addition to traditional variables regarding survival or treatment, eight are included related to patients' degree of health (performance status, patient-reported health status, pain, dysphonia, feeding limitations, oral communication limitations, body image alteration, and need for permanent tracheostomy). Six of them are proposed for tracking *via* HRQoL questionnaires, EQ-5D-3L and EORTC QL-Q H&N43. SCCHN and its treatment can compromise vital functions, such as breathing, swallowing, and speech. Therefore, the disease can lead to significant physical, emotional, and social problems, reducing patients' HRQL. Although the collection of HRQoL and other PROs is scarce in clinical practice, the inclusion of these variables in the standard set was considered key to establishing the impact of the disease from the patients' perspective (24, 25). Due to the lack of resources and the limited knowledge of these questionnaires in clinical practice (26), it was proposed to complete these questionnaires every six months during the first

year and then annually. Moreover, by using the EORTC QL-Q H&N43 questionnaire, information about the impact of the disease and its treatment on nutritional status, such as dysphagia, dental problems, dry mouth, sensory problems, taste/smell alterations, problems with chewing, and weight loss, can also be gathered.

Although some healthcare professionals perceive the use of PROs in clinical practice as time-consuming, a one-year pilot study conducted in the Netherlands demonstrated that the ICHOM standard set could be implemented during routine SCCHN treatment without significantly disturbing the everyday workflow (27, 28). The authors concluded that the collection of PROs is not overly time-consuming; however, it requires *ad hoc* tools and dedicated staff (27, 28).

Providing patient-centered care is essential to move toward high-quality integrated care. Therefore, the inclusion of PROs in the standard set is crucial. Other initiatives have been conducted to promote the use of PROs and patient-reported experiences (PREs) to measure the quality of care, showing that PROs and PREs are promising for measuring and improving the quality and personalization of healthcare in patients with SCCHN (29, 30).

In addition to defining the essential variables for patient follow-up, the experts agreed on those necessary to characterize the patient, the case-mix. The inclusion of these variables is beneficial for benchmarking purposes and for comparing results based on patient profiles.

Data from the implementation of other standard sets in clinical practice has shown benefits from patients' and clinicians' perspectives. On the one hand, the inclusion of PROs in the standard set will allow clinicians to focus on the aspects of the disease that most matter to the patient, and therefore encourage better patient engagement in disease management. Moreover, clinicians can learn from the outcomes data they gather, and from the experience of other healthcare professionals in different settings, the standard set thus becoming a valuable tool for benchmarking (31–34).

This project presents several limitations. This standard set reflects the opinion of a group of 50 experts on the management of SCCHN, four patient representatives (all men) and one patient advocacy group representative. Although no significant differences are expected, different groups of experts and patients, including women, could have agreed on various other recommendations. To minimize this potential bias and ensure national representativeness, participants from four broad geographic areas were involved in the project. Secondly, some health outcomes, for instead data regarding surgical details are finally excluded from the standard set. In this regard, it is important to bear in mind that we aim to achieve a minimum, standard set to ensure that at a minimum these health outcomes are collected. In third place, some relevant variables, such as biomarkers may not have been considered when elaborating on the standard set. To minimize this limitation, and due to the continuing advances in both the knowledge and treatment of this disease, we recommend periodically updating the list of biomarkers for evaluation during patient follow-up. We also suggest that the present standard set be regularly updated.

Although this standard set for SCCHN marks a starting point, several barriers need to be overcome on the road to its successful implementation in the Spanish setting. Namely, the time required for the collection of the health outcomes proposed, the lack of digital tools allowing systematic and automatic PRO measurements (PROMs) compilation, together with limited education and information of patients and clinicians about PROs, have been identified as the main barriers to the implementation of the present standard set (35). Newer platforms for data collection, based on information and communication technologies, may reduce the burden on both patient and clinician, as well as data processing time, thus facilitating the use of PROs in clinical practice (36). It is important to notice that, regardless of the platform used to collect the health outcomes of the standard set, they will be included in the patient's medical record; therefore, the protection of personal data will be guaranteed and will follow the same procedure as the rest of the data in the medical record. Other barriers that must be overcome to ensure the widespread use of this standard set are inherent to the structure of the Spanish national healthcare system (SNHS). Indeed, one of the main characteristics of the SNHS is its heterogeneity: healthcare processes, organizational models as well as information systems differ widely both among and within regions.

Besides addressing these barriers, a further step to promote the integration of the defined standard set into the Spanish healthcare model may involve conducting a pilot implementation study. A pilot study may help establish the feasibility of introducing the standard set in the routine clinical practice, providing insights into the leading resource requirements and organizational challenges to be tackled during implementation.

5 CONCLUSION

The standard set defined may pave the way to standardizing the collection of variables in SCCHN and contribute to promoting the incorporation of patient perspective in SCCHN management. In turn, the information provided through the systematic compilation of this set of health outcomes may allow both clinicians and health policymakers to define strategies aimed at achieving high-quality, patient-centered care.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

VA, GC, DC, JG, RM, FM, AR, and AS: contributed equally to this work with data acquisition and data interpretation. JS, AM, and LL contributed to the conceptualization and design of the study. JS and AM did not participate either as members of Scientific Committee or on the selection of members of the Scientific Committee. MC

contributed to the conceptualization and design of the study, data acquisition, data analysis, data interpretation and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.747520/full#supplementary-material

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miR-22 and miR-205 Drive Tumor Aggressiveness of Mucoepidermoid Carcinomas of Salivary Glands

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Objectives: To integrate mRNA and miRNA expression profiles of mucoepidermoid carcinomas (MECs) and normal salivary gland (NSGs) tissue samples and identify potential drivers.

Material and Methods: Gene and miRNA expression arrays were performed in 35 MECs and six NSGs.

Results: We found 46 differentially expressed (DE) miRNAs and 3,162 DE mRNAs. Supervised hierarchical clustering analysis of the DE transcripts revealed two clusters in both miRNA and mRNA profiles, which distinguished MEC from NSG samples. The integrative miRNA-mRNA analysis revealed a network comprising 696 negatively correlated interactions (44 miRNAs and 444 mRNAs) involving cell signaling, cell cycle, and cancer-related pathways. Increased expression levels of miR-205-5p and miR-224-5p and decreased expression levels of miR-139-3p, miR-145-3p, miR-148a-3p, miR-186-5p, miR-338-3p, miR-363-3p, and miR-4324 were significantly related to worse overall survival in MEC patients. Two overexpressed miRNAs in MEC (miR-22 and

miR-205) were selected for inhibition by the CRISPR-Cas9 method. Cell viability, migration, and invasion assays were performed using an intermediate grade MEC cell line. Knockout of miR-205 reduced cell viability and enhanced *ZEB2* expression, while miR-22 knockout reduced cell migration and invasion and enhanced *ESR1* expression. Our results indicate a distinct transcriptomic profile of MEC compared to NSG, and the integrative analysis highlighted miRNA-mRNA interactions involving cancer-related pathways, including PTEN and PI3K/AKT.

Conclusion: The *in vitro* functional studies revealed that miR-22 and miR-205 deficiencies reduced the viability, migration, and invasion of the MEC cells suggesting they are potential oncogenic drivers in MEC.

Keywords: mucoepidermoid carcinoma, salivary gland tumor, head and neck cancer, oral cancer, transcriptomic analysis, miR22, miR205, microRNA

INTRODUCTION

Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy in major and minor glands, and the most common salivary gland cancer affecting pediatric patients (1). The clinical behavior is variable, ranging from indolent locally infiltrative lesions to highly aggressive and metastatic lesions (2, 3). The widely used histological grade system stratifies MECs into low, intermediate, or high-grade (I, II, or III, respectively) according to histologic characteristics (1, 4–6). Histologic grade and TNM status are commonly used parameters for treatment planning. Treatment of low- and intermediate-grade tumors is based on complete surgical removal of the tumor, while there is no consensus regarding the guidelines for intermediate histologic grade (2, 7–10). In high-grade MEC, the treatment is generally surgery, followed by postoperative radiotherapy. The survival rates for low-grade MEC is over 90% at 10 years, while 70% of intermediate-grade and only 25% of high-grade MEC patients are alive after 10 years (1).

The recurrent chromosome translocation t(11;19) with the resulting *CRTC1-MAML2* fusion oncogene has been described in 60-90% of MECs (10–17). The fusion transcript has been found specific for MECs when comparing with other types of salivary gland tumors (17). *CRTC1-MAML2* has also been considered a prognostic marker (18–20), although its use in prognostication has been questioned (12, 21).

The gene expression profile of MECs has been reported in two studies in which the authors investigated a few MEC cases and compared the differentially expressed (DE) mRNA transcripts with other salivary gland tumors (22, 23).

miRNA expression studies were performed on a few MEC samples focusing on specific gene/miRNA pathways, such as angiogenesis, mast cell activation, and apoptosis (24, 25). In six MEC and three normal salivary gland samples, Binmadi et al. reported 68 DE miRNAs (26) [25]. Among them, miR-302a was the most upregulated and miR-885-5p the most downregulated miRNA (26).

Here, we investigated mRNA and miRNA expression profiles of 35 fresh-frozen MECs and six normal salivary gland tissue samples, followed by an integrative miRNA-mRNA analysis to select potential drivers. In an intermediate grade MEC cell line (UM-HMC-2), we used the CRISPR/Cas9 method to knock down two miRNAs (miR-22 and miR-205) overexpressed in MEC tissues, with the aim of analyzing their role as oncogenic drivers in MEC.

MATERIAL AND METHODS

Patients and Tissue Specimens

We selected 35 MEC samples from patients treated at the A.C.Camargo Cancer Center and Barretos Cancer Hospital, Barretos, São Paulo, Brazil. Two experienced pathologists (FPS and VCA) in salivary gland tumors reviewed the diagnosis of all tumor cases and graded according to Auclair et al., 1992 (4). Demographic, clinical, pathological, therapeutic, and follow-up data were obtained from the patients' medical records (**Table 1**). A reference RNA (Human Universal Reference Total RNA, Clontech, Mountain View, California, USA) was used and hybridized with both tumor RNA and normal salivary gland RNA. Six surrounding normal salivary glands (NSG/control) tissues were removed during surgical procedures of six MEC patients, and they were hybridized with reference RNA to further compare their mRNA and miRNA expressions with MEC's (tumor) mRNA and miRNA expressions. All samples were collected from treatment-naive patients. Written informed consent was obtained from all patients before the sample collection. The National Human Research Ethics Committee approved the study (Protocol #1.380.762/2015).

miRNA Expression Analysis

miRNA expression analyses were performed in 25 out of the 35 fresh-frozen MEC samples and six NSG; no tissue or total RNA was available for analyses in the remaining 10 samples (**Table 1**). Hybridizations were performed using a one-color SurePrint 8X60K Human miRNA platform (G4870A, Agilent Technologies, Santa Clara, CA, USA), as recommended by the supplier. Background correction, quantile normalization, log2

TABLE 1 | Demographic, clinical histopathological, therapeutic and follow-up findings of 35 mucoepidermoid carcinomas patients evaluated by mRNA and miRNA expression analyses.

Characteristics	Number of patients	
	miRNA analysis	mRNA analysis
Age (mean ± SD)	48.7 ± 19.7	47.7 ± 19.8
Gender		
Female	12	20
Male	13	14
Race		
Caucasian	17	26
Asian	1	1
NA	7	7
Anatomical site		
Parotid gland	13	16
Intra oral minor salivary gland and others*	7	8
Hard/soft palate	2	4
Tongue	2	4
Submandibular gland	1	2
cT stage		
T1-T2	8	10
T3-T4	10	14
NA	7	10
cN stage		
N0	13	18
N1	1	2
N2	4	4
N3	0	0
NA	7	10
cM stage		
M0	17	21
M1	1	3
NA	7	10
Tumor Grade		
Low	14	19
Intermediate	6	7
High	5	8
Vital status		
Alive	15	21
Deceased (cause of death MEC)	8	10
NA or dead of other causes#	2	3
Local recurrence		
Yes	6	6
No	18	27
NA	1	1
Treatment		
Surgery	8	13
Surgery and Radiotherapy	15	19
None	2, one received palliative RT	2, one received palliative RT
Distant Metastasis		
Yes	3	5
No	21	28
NA	1	1
Follow-up: median months (IQ range)	49.0 (62.0)	49.5 (59.8)

*NA, Information not available; SD, standard deviation; IQ, Interquartile. *gingiva, maxillary sinus, eye, nasal fossa, nasal septum.*

transformation, and statistical tests were conducted using BRB ArrayTools software v. 4.4.0 (Biometric Research Branch, National Cancer Institute, Bethesda, MD, USA - https://brb.nci.nih.gov/BRB-ArrayTools/index.html). Sequences with more than 10% of MEC and NSG samples presenting undetectable expression (below background signal) were removed. The mean of the probes representing the same miRNA was used in the subsequent steps. miRNAs DE between MEC and NSG groups were identified with a p-value <0.05 (random variance t-test), false discovery rate (FDR) <0.05, and fold change (FC) ≥ 2 and ≤ -2. Supervised hierarchical clustering analysis was performed using 1-minus correlation distance and complete linkage (BRB array tools). Robustness of hierarchical clustering analyses was confirmed using pvclust package (R program) (**Supplementary Figure S1**). Data were deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE199692.

Gene Expression Analysis

Array-based gene expression analysis was performed in 34 out of the 35 fresh-frozen MEC samples and five NSG; one MEC and one NGS sample were excluded based on inferior RNA quality (**Table 1**). Hybridizations were performed using Two-color SurePrint G3 Human Gene Expression Microarray 8x60K (G4851B, Agilent) platform, as previously described (27). Data processing and analyses were carried out using similar parameters described for miRNA profiling (BRB array tools). Identification of DE mRNAs (p-value < 0.001, FDR < 0.05, FC ≥ 2 and ≤ -2) and supervised hierarchical clustering analysis were performed as described above. Pvclust package (R program) was used to confirm the robustness of hierarchical clustering analyses (**Supplementary Figure S1**). The data were deposited in the GEO database (accession number GSE169754).

miRNA-mRNA Integrative Analysis

Target transcripts from the disrupted miRNAs were predicted using the miRWalk 2.0 tool (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), considering only the interactions predicted by at least three of four different bioinformatic algorithms (miRWalk, miRanda, RNAhybrid, and Targetscan). miRNA and mRNA expression data from 24 MEC samples tested by both procedures were integrated based on a significant negative correlation (Pearson correlation, p-value < 0.05) between predicted miRNA-mRNA interactions. Experimentally validated interactions were additionally obtained from the miRTarBase database (28).

Pathway Enrichment Analysis

Pathway enrichment analysis was performed with KOBAS 3.0 (http://kobas.cbi.pku.edu.cn) and pathDIP (http://ophid.utoronto.ca/pathDIP) tools, comprising PANTHER, Reactome, and KEGG databases. Default parameters were adopted in KOBAS 3.0, and only experimentally detected protein-protein interactions were considered in PathDIP. The threshold used in both *in silico* tools was defined as p-value < 0.001 (hypergeometric test) and adjusted p-value < 0.05 (Benjamini and Hochberg method).

Cell Line Culture

Human Mucoepidermoid Carcinoma (UM-HMC-2) cells were isolated from the intermediate grade (stage IVb) parotid gland

MEC of a 59-year-old Caucasian female and cultured according to Warner et al. (29).

CRISPR/Cas9-Mediated Knockout of miRNA-22 and miR-205

miRNA-22 and miR-205 expression in UM-HMC-2 was confirmed using qRT-PCR (data not shown). Then, UM-HMC-2 cells were transfected with pSpCas9(BB)-2A-GF (PX458 expression vector, Addgene plasmid # 48138) expressing CRISPR-Cas9 and sgRNA targeting either miR-22 or miR-205 using Fugene HD transfection reagent (Promega, Madison, WI, USA). This resulted in transient expression of Cas9-sgRNA. Cells transfected with an empty plasmid were used as a control. After 72 hours, cells were sorted for GFP (Green fluorescent protein) positive population using a Sony SH800 cell sorter (Sony Biotechnology, San Jose, CA, USA), and were cloned as single cells per well in a flat bottom 96-well plate. Successfully expanded clones were then screened by capillary sequencing to detect nonhomologous end-joining CRISPR-Cas9 induced gene editing. Clones with predicted out of frame insertions and deletions (indels) were selected and expanded. The predicted effect of the CRISPR editing on miRNAs was assessed using the TIDE tool (30). Details of all sgRNAs and primers used in the experiments, as well as the CRISPR knockout efficiency, are summarized in the supplementary information (**Supplementary Figure S2A** and **Supplementary Table S1**).

qRT-PCR for miRNA

In addition to sequencing, CRISPR knockout of miR-22 and miR-205 was confirmed using qRT-PCR. The miRNA was extracted with miRNeasy Tissue/Cell Advanced Mini Kit (Qiagen, Hilden, Germany) and transcripted to cDNA using miScript II RT Kit (Qiagen) following manufacturer's instructions. The miScript universal primer and miRNA-specific primers for Hsa-miR-22-3p (MS00003220) and hsa-miR-205-5p (MS00003780) were purchased from Qiagen. The relative quantitative expressions were normalized to the endogenous control human RNU-6 (MS00033740) purchased also from Qiagen. Quantitative real-time PCR was performed on Applied Biosystems QuantStudio 5 Real-Time PCR System. qRT-PCR results are summarized in the supplementary information (**Supplementary Figure S2B**).

qRT-PCR for mRNA

In order to study the effect of miR-knockouts, 11 genes were selected and evaluated by qRT-PCR: *PTEN, LAMC1, CADM1, HER3, MYCBP, SNAI1, YAP1, CD147, SMAD4, ESR1* (*ESR1*) and *ZEB2*. One thousand ng of the total RNA was used for cDNA synthesis. Synthesis was done using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Two nanograms of cDNA was used for performing qRT-PCR with the Fast SYBR Green Master Mix (Thermo Fisher Scientific) as per the manufacturer's instructions. The relative quantitative expression was normalized to the endogenous control *GAPDH*. The primers were purchased from Metabion (Planegg, Germany) and the sequences are summarized in the supplementary information (**Supplementary Table S1**). Quantitative real-time PCR was performed on Applied Biosystems QuantStudio 5 Real-Time PCR System.

Cell Viability Assay

A CellTiter-Glo (CTG) 2.0 Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to determine the effect of miR-22 and miR-205 on the cells' viability. Briefly, 100 µL of cell suspension was dispensed in the Perkin Elmer ViewPlate-96 microplate with a clear flat bottom and black well walls (Perkin Elmer Inc., Waltham, MA, USA) for a final concentration of 1000 cells per well. After 72 hours, 100 µL of the CellTiter-Glo reagent was dispensed into the wells, and the luminescence reads were measured using a PHERAstar plate reader (BMG Labtech, Ortenberg, Germany).

Scratch Wound Cell Migration and Invasion Assays

IncuCyte 96-well ImageLock Microplate wells (Sartorius, Göttingen, Germany) were coated with 300 µg/mL Myogel for migration and invasion assays (31). The cells were seeded at a density of 25,000 cells per well in 100 µL of complete medium for both assays. After 24 hours at 37°C, a 96-pin IncuCyte WoundMaker Tool (Sartorius) was used to make uniform wounds on the confluent monolayer of the cell. The wells were washed two times with media, and 100 µL of complete medium was added. For the invasion plate, 50 µL of Myogel-collagen gel (2.4 mg/mL Myogel, 0.8 mg/mL type I rat tail collagen) (Corning Incorporated, Corning, NY, USA) was added on top of the cells. After the gel was solidified, 50 µL of media was added, and the plates were transferred to an incubator. The wound closing was monitored automatically every 2 hours for two days using IncuCyte S3 Live-Cell Imaging System (Sartorius). Analysis of wound closing (width of the wound) was performed using Matlab. Mathematical function decorrelation was used to make the cells' intensity substantially higher than the background.

Spheroid Invasion Assay

The spheroid invasion assay was done according to Naakka et al. (32). The UM-HMC-2 cells were seeded at a concentration of 1000 cells per well in 50 µL of the complete medium using a U-shaped ultra-low attachment 96-well plate (Corning, New York, USA) and incubated for four days. Next, the spheroids were embedded in 50 µL Myogel-fibrin gel containing 0.5 mg/mL Myogel, 0.3 U/mL thrombin (Sigma-Aldrich), 33.3 mg/mL aprotinin (Sigma-Aldrich), and 0.5 mg/mL fibrinogen (Merck). After the Myogel-fibrin matrix (30 min) solidification, 100 µL of complete medium was added to the wells.

Images of the spheroids were captured daily using Nikon Eclipse TS100 Inverted Microscope (Nikon, Minato, Tokyo, Japan) at 4x magnification. Analysis of the spheroid invasion area and length of the longest branch was performed using ilastik (freeware) and Fiji ImageJ 1.51 software (33).

Statistical Analysis

All *in vitro* assays were repeated at least three times, each performed at least in triplicate. Statistical analyses were carried out with SPSS v.25.0 (IBM Corporation, Chicago, IL, USA) and the GraphPad Prism software (v. 6.0; GraphPad Software Inc., La Jolla, CA, USA). Student's T-test or One-way ANOVA followed by Bonferroni correction was used in posthoc analysis. A p-value < 0.05 was considered as statistically significant. Figures were

created with Origin 2018b graphing software (OriginLab Corporation, Northampton, MA, USA).

Overall survival analysis was performed using the Kaplan-Meier estimator with the log-rank test in IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp. Armonk, NY, USA). The miRNA expression values were dichotomized below and above the median (p-value < 0.05). A random variance t-test using BRB ArrayTools software (v. 4.4.0) was applied to investigate differences in the miRNA expression in relation to the histological grade, lymph node, and distant metastasis (p-value < 0.05, FDR < 0.05).

RESULTS

The mean age of whole patient group was 46.9 ± 20.2 years (range 12 to 82 years old). Female patients were more frequently affected by MEC than males (ratio 1.4:1). Parotid was the most common anatomical site, followed by minor salivary glands of the palate and other sites. Twenty cases presented with low histologic grade, seven with intermediate-grade, and nine with high-grade at diagnosis. T3-T4 tumors at diagnosis were found in 14 cases. Six patients presented lymph node involvement at diagnosis and three patients presented distant metastases at diagnosis. Twenty patients were treated with surgery and radiotherapy, while 14 received surgery only. Follow-up time ranged from 4 to 188 months (median 49,5 months). Demographic, clinical, histopathological, therapeutic and follow-up features are detailed in **Table 1**. The study design, methodologies, and the foremost results are presented in **Figure 1**.

miRNA and mRNA Expression Profile of MEC

After excluding uniformly low expressed miRNAs in MEC and NSG samples, 530 miRNAs and 19,911 mRNAs were considered for further analysis. We found 46 DE miRNAs (18 overexpressed

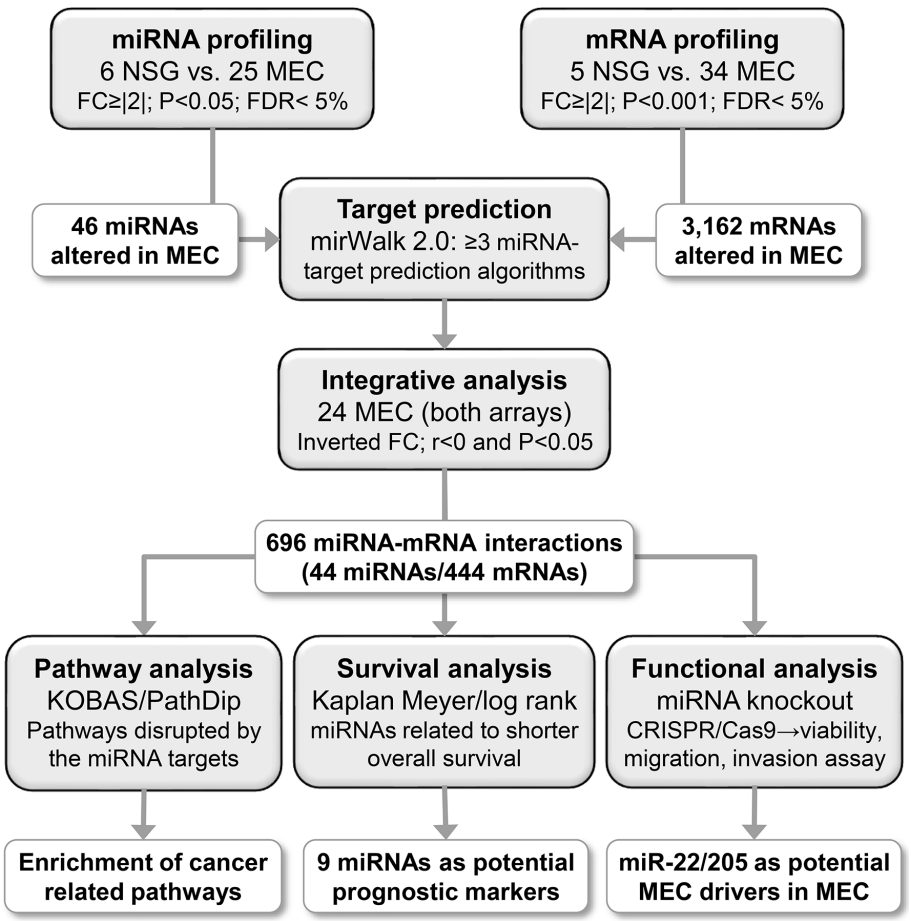

FIGURE 1 | Study design and main results obtained from the miRNA and mRNA expression analyses. First, a miRNA and mRNA global expression analyses revealed 46 miRNAs and 3,162 mRNAs differentially expressed in MEC compared to SNG. An integrative analysis was carried out using predicted miRNA-mRNA interactions, generating a network containing 44 miRNAs and 444 mRNAs (696 interactions). The target genes were associated with cancer-related pathways, and nine miRNAs were associated with shorter overall survival. A knockout assay was performed for miR-22 and miR-205 (CRISPR/Cas9), resulting in viability, migration, and invasion reduction, which indicate their role as putative cancer drivers in MEC.

and 28 underexpressed) in MEC (**Supplementary Table S2**). The most significant (P adjusted ≤ 0.005) overexpressed miRNAs included miR-21-5p (FC=10.2), miR-22-3p (FC=2.0), miR-181a-5p (FC=2.9), miR-205-3p (FC=14.7), and miR-224-3p (FC=5.3). The miR-363-3p (FC =-16.3), miR-625-5p (FC =-18.5), miR-885-5p (FC =-10.7), miR-892b (FC =-2.7), and miR-1288-3p (FC =-2.7) were significantly underexpressed (**Figure 2**). A similar approach used for mRNAs unveiled 3,162 mRNAs differentially expressed in MEC (1,488 overexpressed and 1,674 underexpressed (**Supplementary Table S3**).

Supervised hierarchical clustering analysis based on the DE transcripts revealed two clusters in both miRNA (**Figure 3A**) and mRNA (**Figure 3B**). Although these two main clusters completely separated MEC from NSG samples, no association was observed when comparing the clinical-pathological parameters (histological grade, lymph node involvement, and distant metastasis) with the clusters generated by both miRNA and mRNA analysis.

The miRNA-target prediction analysis resulted in 20,816 miRNA-mRNA putative interactions. The integrative analysis revealed a miRNA-mRNA network comprising 696 negatively correlated interactions and inverted FCs (44 miRNAs and 444 mRNAs) (**Supplementary Table S4**). The main biological pathways uncovered by miRNA targets corroborated by the integrative analysis were cell signaling, cell cycle, and cancer-related pathways (**Table 2**).

Lower expression levels of miR-582-5p, miR-3125, and miR-4324 were found in high-grade MEC compared to low and intermediate grades (**Supplementary Figure S3**). Increased expression levels of miR-205-5p and miR-224-5p (both overexpressed in MEC) and decreased expression levels of miR-139-3p, miR-145-3p, miR-148a-3p, miR-186-5p, miR-338-3p, miR-363-3p and miR-4324 were significantly related to worse overall survival in MEC patients (**Figure 4**).

Among the list of differentially expressed miRNAs, we selected miR-22 and miR-205 for functional assays for the following reasons: they were significantly overexpressed (adjusted p-value <0.005) (**Figure 2**), presented high interactivity in the integrative analysis (>10 underexpressed mRNA predicted targets negatively correlated with the miRNA expression) (**Supplementary Table S4**), and showed clinical association with worse prognosis (increased miR-205 expression was associated with shorter overall survival) (**Figure 4**).

Knockout of miR-205 Decreases MEC Cell Viability While the Knockout of miR-22 Reduces MEC Cell Migration and Invasion

We explored the use of the CRISPR-Cas9-based method to knockout miR-22 and miR-205 in MEC. Cell viability, migration, and invasion assays were performed in the MEC cell line UM-HMC-2. The cell viability was the lowest in the miR-205 knockout, followed by miR-22-knockout cells, but with no statistical significance (**Figure 5A**).

The scratch wound migration assay showed that miR-22 and miR-205 knockouts reduced cell migration. The effect was the same in both knockout cell lines compared to the empty vector, but miR-22-knockout cells migrated significantly slower than the control cells (**Figures 5B–D** and **Supplementary Figure S4A**). The cell lines showed different invasion speeds, and the knockouts invaded slower in both scratch wound invasion (**Figures 5E–F** and **Supplementary Figure S4A**) and spheroid invasion assays (**Figures 5G–K** and **Supplementary Figure S4B**). Both miR-22 and miR-205 knockout cell lines invaded slower than the cell line with empty gRNA vectors. However, the effect was statistically significant only when miR-22-knockout cells were compared to the empty vector in the spheroid invasion assay.

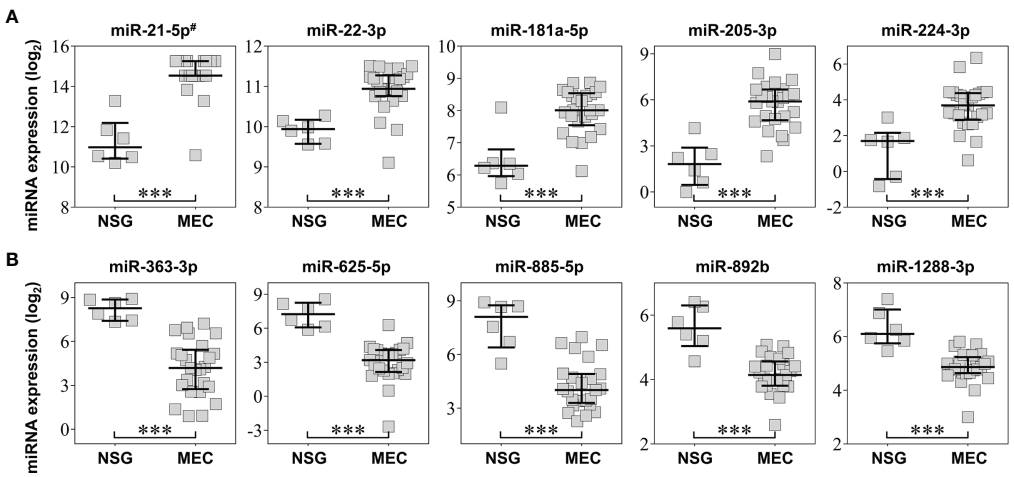

FIGURE 2 | Top five most significant overexpressed **(A)** and underexpressed **(B)** miRNAs obtained in the microarray analysis. The error bars and middle line represent the interquartile range and median, respectively. NSG: surrounding normal salivary gland tissues; MEC: mucoepidermoid carcinoma tissues. #miR-21-3p was omitted (both mature sequence from miR-21 precursor were highly significant). ***P < 0.001 (t test).

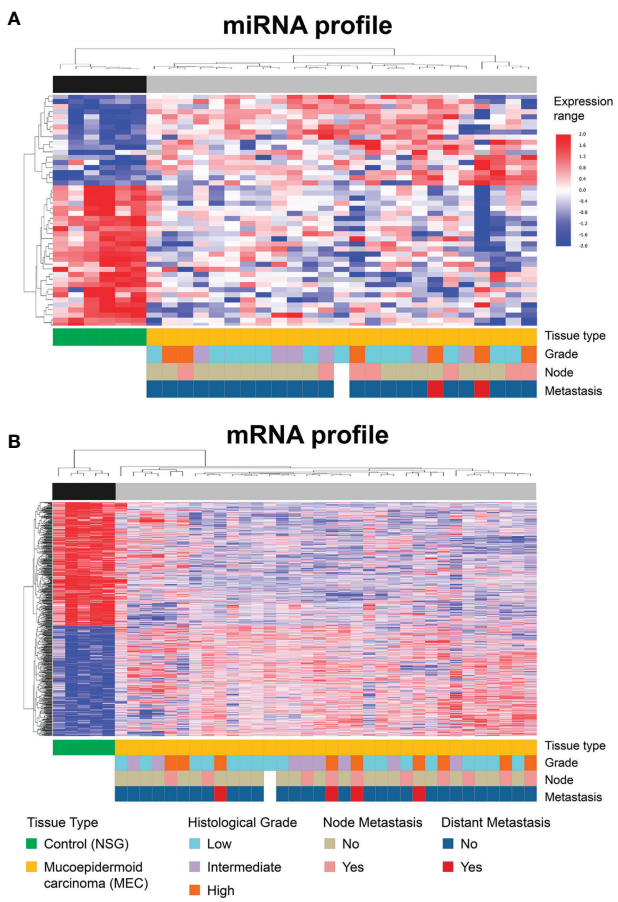

FIGURE 3 | Supervised hierarchical clustering analysis considering the miRNA **(A)** and mRNA **(B)** expression profiles. The dendrograms show a complete separation between MEC and NSG samples according to the 47 miRNAs **(A)** and 3,162 mRNA differentially expressed **(B)**. The samples are represented in columns and miRNAs/genes in rows.

Knockout of miR-22 Induces ESR1 and Knockout of miR-205 Induces ZEB2 Expression

In order to understand the mechanism behind the effect of miR-22 and miR-205 knockout on MEC cell behaviour, we studied the expression of specific molecules: *PTEN, LAMC1, CADM1, HER3, MYCBP, SNAI1, YAP1, CD147, SMAD4, ESR1* and *ZEB2* which, based on the literature, are known to be targets either for miR-22 or miR-205. We reported significant differences in two of the targets: estrogen receptor alpha (*ESR1*) for miR-22 and zinc finger E-box-binding homeobox 2 (*ZEB2*) and miR-205 (**Figure 6**). These molecules influence cell proliferation, migration and invasion (34–36). As expected, miR-22 knockout cells have significantly higher expression of *ESR1*, and miR-205 knockout cells have significantly higher expression of *ZEB2* compared with the empty vector.

DISCUSSION

Varied clinical behavior and multiple histologic grading systems have challenged pathologists in prognostication of MEC and clinicians in making an appropriate treatment decision for the patients (37). Moreover, the differential diagnosis between a salivary gland MEC and other lesions, such as salivary duct cyst, cystadenoma, or glandular odontogenic cyst may be difficult in some situations. In particular, small incisional biopsies are often problematic in the diagnostic workup. The presence of the *CRTC1-MAML2* fusion gene can be helpful for the diagnosis of MEC, but it is not found in all cases of MEC, and there is contradiction about some benign conditions (38–40). Mucoepidermoid carcinomas of the salivary gland are poorly explored at the molecular level. Therefore, genetic studies can unravel diagnostic, prognostic, and predictive markers, as reported in several tumor types.

TABLE 2 | Biological pathways enriched (P value < 0.001 and P adjusted < 0.05) by the genes detected in the miRNA-mRNA integrative analysis (KOBAS 3.0 and Pathdip *in silico* pathway tools).

Biological Pathways	Database	KOBAS 3.0		Pathdip*	
		P value	P adj	P value	P adj
Signal Transduction	Reactome	4E-12	4E-09	6E-05	8E-03
Post-translational protein modification	Reactome	1E-08	4E-06	2E-04	1E-02
Membrane Trafficking	Reactome	2E-07	2E-05	5E-04	2E-02
Diseases of signal transduction	Reactome	3E-06	2E-04	9E-05	8E-03
Signaling by Rho GTPases	Reactome	8E-06	4E-04	1E-04	1E-02
EPH-Ephrin signaling	Reactome	2E-05	8E-04	9E-05	8E-03
Cell Cycle	Reactome	2E-05	9E-04	4E-05	9E-03
RHO GTPase Effectors	Reactome	3E-05	1E-03	8E-06	3E-03
Proteoglycans in cancer	KEGG	2E-04	4E-03	5E-05	8E-03
Cell Cycle, Mitotic	Reactome	2E-04	4E-03	2E-04	1E-02
DNA Double-Strand Break Repair	Reactome	2E-04	5E-03	1E-03	3E-02
EPH-ephrin mediated repulsion of cells	Reactome	4E-04	8E-03	6E-04	2E-02
MicroRNAs in cancer	KEGG	9E-04	1E-02	6E-05	7E-03

*KEGG, Kyoto Encyclopedia of Genes and Genomes; *Experimentally detected protein-protein interactions.*

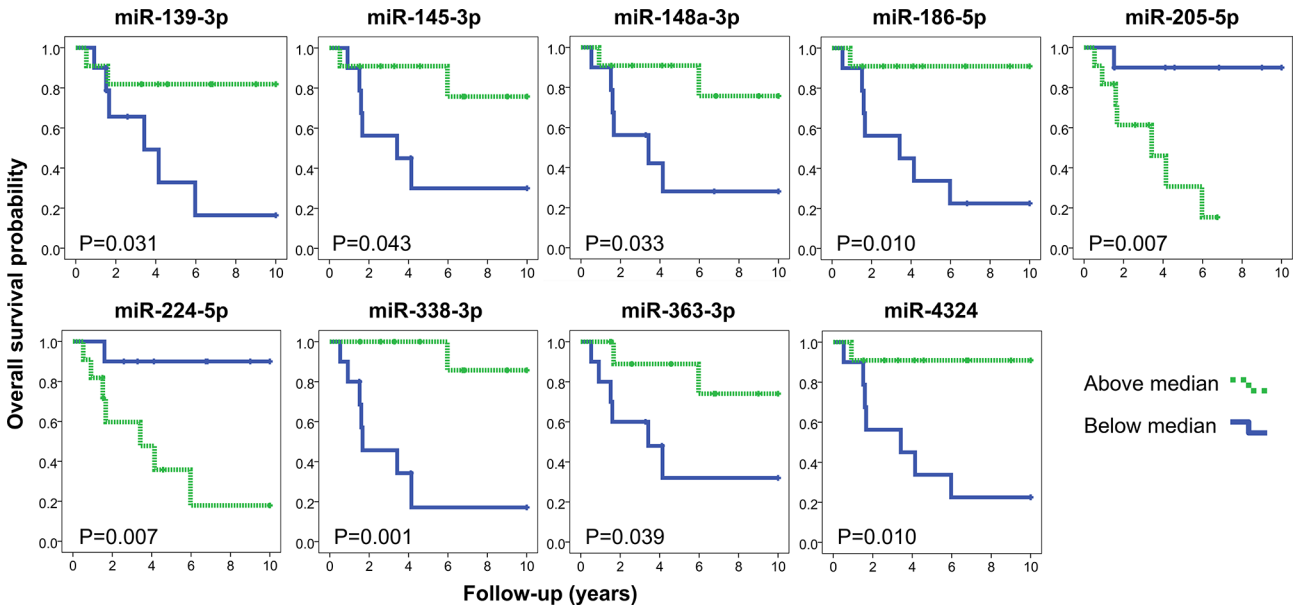

FIGURE 4 | Kaplan-Meier representation of overall survival according to the expression levels of nine miRNAs (log rank test P<0.05). The quantifications obtained by the microarray analysis were stratified in below (blue) and above (green) the median values. Note: miR-224-3p was omitted (both mature sequences from mir-224 precursor were associated with overall survival).

In the present study using large-scale expression analyses, we found 46 miRNAs and 3,162 mRNAs differentially expressed compared to normal salivary glands. In agreement with our present miRNA findings, a previous MEC study reported that miRNA-205 and miRNA-22 were amongst the highest overexpressed miRNAs in MEC, while miRNA-885-5p and miRNA-375 were downregulated (26).

Two earlier studies have investigated global gene expression in MEC (22, 23), but none of the genes reported were found in our analysis. A possible explanation for this discordance may be the small number of MEC cases (2 and 6) investigated in the earlier studies and/or the different methodological strategies. For instance, Leivo et al. (22) focused on comparing different histological types of salivary gland malignancies, which might explain the disparities compared with our findings.

Although we could not investigate the *CRTC1-MAML2* status in our sample set due to a lack of sample material, we observed a decreased *CRTC1* expression level. In MEC, the *CRTC1-MAML2* gene fusion activates *CREB/Cyclic* AMP related genes and possibly the Notch pathway (11, 41–45). Recently, Chen et al. (2021) (46) suggested that deregulated *p16-CDK4/6-RB* signaling is a cooperating event in the progression of MEC with the *CRTC1-MAML2* fusion. The authors also suggested that *EGFR* and *CDK4/6* inhibitors are potentially useful to treat MEC patients.

An integrative analysis was conducted to elucidate the role of miRNAs and their mRNA targets and the core genes and pathways involved in MEC. We found 669 miRNA-mRNA interactions (44 miRNAs and 444 mRNAs) involving cancer-related pathways such as miRNAs in cancer, cell cycle and signal transduction, ERK/MAPK signaling, EIF2 signaling, PI3K/AKT, among others. These findings provide supportive evidence for the detection of drivers involved in MEC pathogenesis. A set of these transcripts was associated with poor prognostic features, such as high histological grade. For instance, a decreased expression of miR-582-5p in MEC was related to high-grade tumors. Previously, miRNA-582-5p downregulation was described in salivary gland tumors (47, 48), and its induction inhibited invasion and migration in salivary adenoid cystic carcinoma (AdCC) (48). We found that the target of this miRNA, *EZH2*, was overexpressed and related to high-grade MEC (**Supplementary Table S4** and **Supplementary Figure S3**). *EZH2* is a member of the polycomb group of proteins involved with transcription regulation through chromatin remodeling (49). Increased EZH2 protein expression has been reported in MEC, myoepithelial carcinoma of salivary glands, and AdCC (50–52). In AdCC, increased EZH2 expression was associated with a worse prognosis.

Significantly decreased miR-4324 expression was detected in our high-grade MEC compared to low/intermediate-grade tumors, and it was also associated with shorter overall survival. miR-4324 has been shown to be underexpressed in a subset of *PTEN* deficient breast cancer patients with exceedingly poor prognoses (53). *PIK3CA* and *PTEN* inactivating mutations are frequent events in high-grade MEC (54). Interestingly, a highly predicted interaction between miR-205-3p and *PLAC8* from the PI3K pathway was observed in our integrative analysis. A recent study demonstrated that *PLAC8* contributes to cell proliferation and suppresses cell apoptosis in breast cancer by activating the PI3K/AKT/NF-κB pathway (55).

Based on established criteria, including increased expression levels, high interactivity in the integrative analysis, and

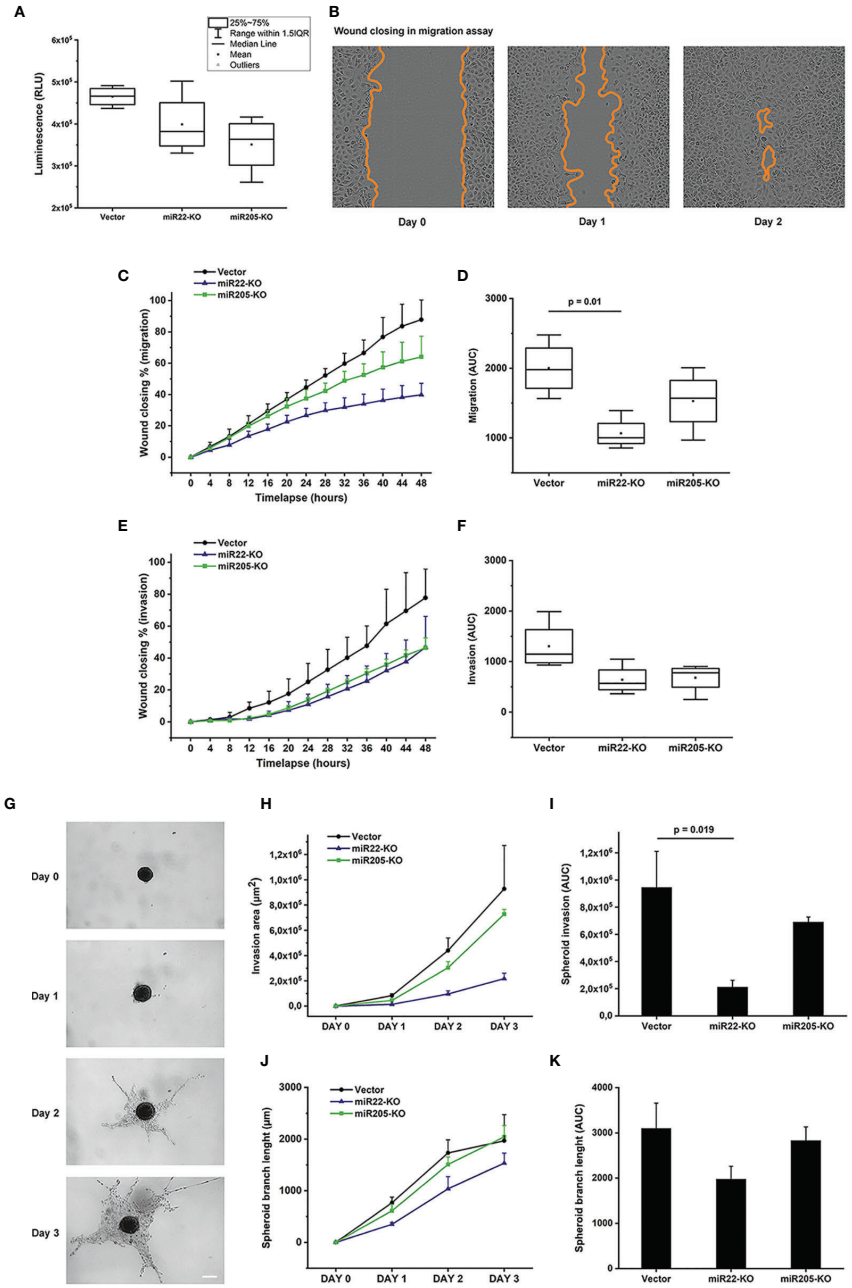

FIGURE 5 | Cell viability, migration and invasion assays performed using the UM-HMC-2 cell line. **(A)** UM-HMC-2 cells were cultured for three days and the cell viability was measured using luminescent cell viability assay. Although not statistically significant, the cell viability was decreased in both miR22- and miR205-knockout cell lines compared to the cell line transfected with an empty plasmid vector. **(B–D)** UM-HMC-2 cells were cultured on Myogel matrix and cell migration was evaluated using scratch wound cell migration assay. **(B)** Representative image of migration distance at 0, 24, and 48 hours after wounding. **(C, D)** Quantification of cell migration in scratch wound assay. miR22- and miR205-knockout cell lines migrated slower than the vector cell line. Statistically significant difference was denoted between vector and miR22-KO cell lines. **(E, F)** UM-HMC-2 cell invasion through Myogel-collagen in scratch wound cell invasion assay. UM-HMC-2 cells were cultured in Myogel-collagen matrix, and cell invasion was evaluated using scratch wound cell invasion assay. miR22- and miR205-knockout cell lines invaded slower than vector cell line (p-value > 0.05). **(G–K)** UM-HMC-2 cell invasion through Myogel-fibrin in spheroid invasion assay. Cells were cultured in U-shaped ultra-low attachment 96-well plate wells and embedded in Myogel-fibrin matrix. Spheroids were observed under a light microscope and the invasion area and the spheroid branch length were analyzed using ilastik and ImageJ software. **(G)** Representative images of spheroid invasion at different time points. Scale bar = 200 µm (Original magnification X4). **(H, I)** Quantification of cell invasion in 3D spheroid invasion assay. Knockout of miR22 and miR205 reduced tumor cell invasion. Difference between vector and miR22-KO cell lines reached statistical significance. **(J, K)** Quantification of spheroid branch length revealed that miR22- and miR205-knockout cell line spheroids did not extend as far as vector cell line (p-value > 0.05). Data are presented as means ± SD of 3-4 independent experiments, each at least in triplicate. $p < 0.05$ is considered as significantly different compared to vector control.

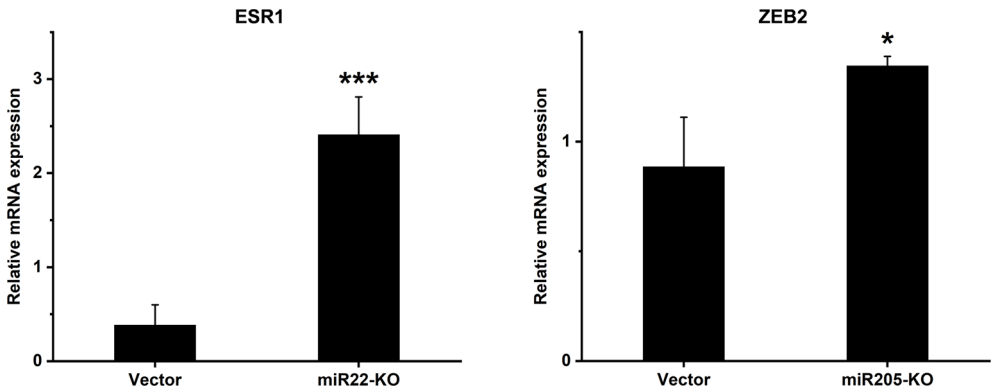

FIGURE 6 | mRNA expression levels of selected genes after miR-knockout. Expression levels of *ESR1* in miR22-KO and *ZEB2* in miR205-KO cell lines were analysed using qRT-PCR. The relative mRNA levels are shown after normalization to GAPDH. *ESR1*, Estrogen receptor alpha; *ZEB2*, Zinc finger E-box binding homeobox 2. Data are presented as means ± SD. *p ≤ 0.05, *** ≤ 0.001.

association with clinical parameters, we selected two miRNAs, miR-205 and miR-22, for functional assays. These two miRNAs were among the highest overexpressed miRNAs in previously described MEC cases (26). miR-205 was one of the most significantly overexpressed miRNAs, and it was associated with shorter overall survival in our MEC cases. Overexpression of this miRNA has been reported in several cancers, including AdCC and head and neck squamous cell carcinomas (56–58). A previous study suggested that miR-205-5p targets *PTEN* to regulate the epithelial mesenchymal transition through the PI3K/AKT pathway (58).

Since miR-22 was one of the highest overexpressed miRNAs in MEC, it was selected for knockdown and functional experiments. Dysregulation of this miRNA has been reported in several tumor types (59) and implicated in the regulation of cell growth, cell cycle, apoptosis, and invasion (60, 61). *MYC* and *PI3K/AKT* can induce miR-22 gene expression, which in turn targets *PTEN* (62). Since PTEN is a repressor of AKT, miR-22 could act as a key element in a positive feedback of the *PI3K/AKT* pathway to cause downregulation of *PTEN* (59). As previously described in MEC (13), this miRNA also induces chromosomal instability (63). Knockdown of miR-22 showed a consistent reduction of viability, migration, and invasion of MEC cells. However, the effect on migration and invasion was stronger and seems not to be as a result of reduced viability which was only mild and not significant.

Previous studies have reported that miR-22 represses *ESR1* expression in breast cancer and lead to a reduction in estrogen signaling (34). In line with that, we showed that the miRNA-22 knockout increased *ESR1* expression levels. *ZEB2* was reported to negatively correlate with miR-205 levels in esophageal squamous cell carcinoma cells (35) and silencing of *ZEB2* lead to suppressed cell viability, migration, and invasion in laryngeal squamous cell carcinoma cells (36). Our data showed an upregulation of *ZEB2* in miR-205-knockout cells which is in line with the reports above. Additionally, *ZEB2* has been shown to directly bind to the E-cadherin promoter and repress its transcription (64). Loss of E-cadherin is one of the main initiation events of epithelial to mesenchymal transition (EMT) and thus plays an important role in cancer progression. The biological mechanism behind these actions remains to be elucidated in future studies.

CONCLUSION

Although we investigated a limited number of cases, we described a transcriptomic profile distinguishing MEC from normal salivary glands. The integrative analysis highlighted miRNA-mRNA interactions, and cancer-related pathways were described. Comparison with other studies using similar strategies was limited due to the absence of available miRNA-mRNAs expression data in public databases. However, our list of differentially expressed miRNAs-mRNAs revealed that PTEN and PI3K/AKT pathways were altered in MEC. Our *in vitro* functional assays indicate that miR-22 and miR-205 deficiencies reduce cell viability, migration, and invasion in a MEC cell line by enhancing the expression of *ZEB2* and *ESR1* mRNAs. Taken together, our findings suggest that these dysregulated miRNAs have a pathogenic role in MEC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The National Human Research Ethics Committee (Protocol #1.380.762/2015). Written informed consent to

participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Study concept and design: FP-S, TS, and SR. Data Acquisition: EN, MB-F, HK, KT, and AB. Quality control and data algorithms: MB-F, SA, FM, IS, and SL. Data analysis and interpretation: FP-S, EN, MB-F, and FM. Statistical analysis: MB-F, SA, FM, and IS. Manuscript preparation: FP-S and EN. Manuscript editing: EN, FP-S, TS, and SR. Manuscript review: CS-N, RC, LK, AM, VA, IL, TS, SR, and AA-S. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.786150/full#supplementary-material

Supplementary Figure S1 | Bootstrap analysis to estimate the cluster stability. B = 1000 bootstraps conducted with pvclust package (R program).

Supplementary Figure S2 | (A) CRISPR knockout efficiency and indel spectrum. The predicted effect of the CRISPR-editing on miRNAs was assessed using TIDE online tool by The Netherlands Cancer Institute, Amsterdam, Netherlands (https://tide.nki.nl/). **(B)** qRT-PCR assay reveals down-regulation of miR-22-3p ($p < 0.008$) and miR-205-5p ($p < 0.004$) expression in knockout cells compared to vector control.

Supplementary Figure S3 | Differentially expressed miRNAs according to the histological grade. miR-582-5p (FC= -5.1; P=0.0001; FDR =0.0066), miR-4324 (FC= -3.7, P=0.0019, FDR=0.0305) and miR-3125 (FC=-2.1, P=0.0031, FDR=0.0305) were all underexpressed in high-grade mucoepidermoid salivary gland carcinoma compared to low/intermediate grade. ***$P < 0.001$, **$P < 0.01$ (t test).

Supplementary Figure S4 | UM-HMC-2 cell migration and invasion assays. **(A)** Representative images of UM-HMC-2 cell migration and invasion distance at 0, 24, and 48 hours in wound scratch wound assay. **(B)** Representative images of UM-HMC-2 cell invasion through Myogel-fibrin in spheroid invasion assay at different time points. Scale bar = 200 μm (original magnification X4).

Supplementary Table S1 | Primers used in this study. Oligonucleotide pairs for construction of gRNA expression plasmids, primer sequences used to amplify the target site before the Sanger sequencing and primers for target gene qRT-PCR (F, forward; R, reverse).

Supplementary Table S2 | Differentially expressed miRNA in salivary gland mucoepidermoid carcinoma (MEC) compared to normal salivary gland (NSG) tissues.

Supplementary Table S3 | Differentially expressed mRNAs in MEC compared to non-neoplastic salivary gland tissues (excel file).

Supplementary Table S4 | MicroRNA and target-mRNA interactions retrieved from the integrative analysis, comprising the transcripts differentially expressed in MEC (excel file).

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Prevalence of Transcriptionally Active HPV Infection in Tumor-Free Oropharyngeal Tissue of OPSCC-Patients

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Objectives: The natural history of HPV-related oropharyngeal squamous cell carcinoma (OPSCC) is still largely unknown. Since reports of second primary tumors (SPTs) in patients with HPV-related OPSCCs are increasing, a multifocal HPV infection, hinting a «virus-induced field effect», has been hypothesized. This study aimed to investigate the HPV-prevalence in normal appearing oropharyngeal tissue in patients with OPSCCs.

Materials and Methods: 49 OPSCC patients undergoing panendoscopy were prospectively enrolled. Tumor specimens and biopsies of normal appearing oropharyngeal tissue adjacent to and distant from the index OPSCC underwent histopathological examination, $p16^{INK4A}$ immunohistochemical staining, HPV DNA and mRNA-detection. Patient characteristics and follow-up data on SPTs were obtained.

Results: 26 of 49 (53%) OPSCC were positive for HPV DNA and $p16^{INK4A}$. HPV mRNA was detected in 23 of 26 (88%) of these tumor samples. HPV DNA was detected in 36% adjacent mucosa and in 17% distant mucosa samples and only in patients with an HPV-related index OPSCC. HPV mRNA could not be detected in tumor-free distant and adjacent mucosa samples. No evidence of association between HPV detection in normal appearing mucosa and development of second primary tumors was found.

Conclusions: HPV was detectable but not transcriptionally active in adjacent/distant tumor-free oropharyngeal tissue. This suggests that a multifocal HPV infection, hinting a «virus-induced fielcd cancerization», may not be pertaining to HPV-related OPSCC.

Keywords: head and neck cancer, oropharynx cancer, human papillomavirus, second primary neoplasms, human papillomavirus DNA tests

1 INTRODUCTION

A transcriptionally active infection with high risk human papillomavirus (HR-HPV) is now established as a major risk factor for the development of oropharyngeal squamous cell carcinomas (OPSCCs) (1, 2). HPV-related OPSCCs are associated with a better treatment response and improved outcome compared to their tobacco- and alcohol-induced counterparts (3, 4). Moreover, compelling evidence demonstrates a lower risk of developing second primary tumors (SPT) in these patients, possibly contributing to their superior overall and disease-free survival (5–8). SPTs represent, in fact, one of the leading causes of death in head and neck cancer patients and typically arise within the upper aerodigestive tract (UADT) (9). The development of SPTs has been linked to the concept of «field cancerization», which describes multifocal alterations of mucosa "fields" following a progressive, alcohol- and tobacco-induced accumulation of adverse genetic modifications (10). Nevertheless, some cases of synchronous and metachronous HPV-driven SPTs have been recently described (11–17), raising the question of a possible, multifocally persistent oncogenic infection with HR-HPV in the oropharyngeal mucosa.

The mechanism underlying the transformation from a transient oropharyngeal HPV infection to virus-induced cancerization is still largely unknown (18). In fact, while HPV DNA has been frequently detected in dysplastic tonsillar epithelium (19), several studies reported a moderate (6.9% - 13.1%) to low (0 - 6.3%) HR-HPV prevalence in respective oral gargles (20, 21) and non-malignant tonsillar tissue (22–26). Thus, detection of transcriptionally active HR-HPV in normal appearing mucosa may indicate areas of «virus-induced field cancerization».

The aim of this study was to prospectively and systematically assess the prevalence of a transcriptionally active HR-HPV infection in the normal appearing mucosa adjacent to and distant from the tumor in patients with OPSCCs and its potential impact on development of SPTs.

2 MATERIALS AND METHODS

2.1 Patients and Sample Collection

Details on the patients' cohort have been previously published (27). Briefly, patients with oropharyngeal squamous cell carcinoma (OPSCC) undergoing panendoscopy were prospectively enrolled at Kantonsspital St. Gallen. The study was approved by the local ethics committees (EKSG 09/124') and written informed consent was obtained from all patients prior to study entry. Patient characteristics and follow-up data were obtained from questionnaires and clinical charts. Follow-up time (months) was reported after completion of therapy. In the present analysis, second primary tumors (SPT) from the upper aerodigestive tract (UADT) comprising the oral cavity, oropharynx, hypopharynx, larynx and esophagus were considered. Tumors that have been diagnosed more than six months apart from time of diagnosis of the index tumor were defined as metachronous (11).

During panendoscopy, iodine solution for mucosal disinfection was used. Biopsies (approximately 3 mm) were taken from the tumor, from the normal appearing mucosa adjacent to the tumor and from macroscopically normal appearing contralateral tonsil tissue at least 10 mm distant from the tumor. In order to avoid cross-contamination sampling was performed in the following sequence: contralateral – adjacent – tumor tissue and instruments were replaced after each biopsy. Tumor biopsies were primarily used for diagnostic purposes; fractions of these specimens were preserved for further molecular analysis in the context of this study. HPV tumor status by HPV DNA and $p16^{INK4A}$ immunohistochemical staining (IHC) was assessed in diagnostic biopsies and in surgical specimens of the tumor, if resection was performed.

2.2 Histopathological Examination and $p16^{INK4A}$ Immunohistochemical Staining

Tissue specimens were available as formalin-fixed paraffin-embedded (FFPE) blocks. FFPE tissue sections were prepared using a microtome. The first and the last 4 µm section from each biopsy were prepared for hematoxylin/eosin (HE) staining. In between, 3 x 10 µm curls were sectioned for DNA and RNA isolation, respectively, and one 4 µm section was prepared for $p16^{INK4A}$ immunohistochemical staining. During sectioning of FFPE tissues, an established cleaning protocol was applied in order to prevent cross-contamination between samples. The microtome was extensively cleaned with acetone, ethanol and RNase AWAY® (Molecular Bio-Products, Inc., San Diego, CA, USA) before and after each biopsy and blades and gloves were replaced. After five to ten patient tissues, a mouse brain tissue was processed to monitor potential cross-contamination.

Histopathological analysis for presence of tumor cells or dysplasia was independently performed by two pathologists (KI, NJR) on hematoxylin/eosin stained sections. $p16^{INK4A}$ immunohistochemical staining was performed on an automated staining system (Ventana BenchMark ULTRA, Roche-Ventana Medical System, Tucson, AZ, USA) according to the manufacturer's instructions. After pretreatment with Cell Conditioner Solution (CC1) for 48 min, mouse monoclonal antibody (anti-$p16^{INK4A}$, clone E6H4, ready to use) was applied for 4 min (Roche Diagnostics, Rotkreuz, Switzerland). $p16^{INK4A}$-positivity, was defined as unequivocal nuclear and cytoplasmic staining in at least 70% of the tumor cells (28).

2.3 Isolation of Nucleic Acids

Genomic DNA was isolated from tissue sections by incubating for 16 h at 56°C in 200 µl of proteinase K solution (1 mg/ml, 45mM Tris-HC, 0.9mM EDTA, 0.45% Tween 20). This incubation was followed by enzyme inactivation for 10 min at 72°C and centrifugation. The aqueous phase containing the DNA was transferred into a nuclease-free tube. To reverse potential cross-linking, the isolated DNA was incubated for 20 min at 90°C, centrifuged and transferred into a new tube. For PCR, 5 µl of DNA was used. Total RNA was isolated using the Pure-Link FFPE Total RNA Isolation Kit (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with an additional DNase treatment for 15 minutes at room temperature prior to elution in 50 µl RNase-free water. The PCR was performed with 1 µl of RNA.

2.4 HPV DNA and RNA Assays

HPV DNA was detected in biopsies using a previously described multiplex HPV genotyping (MPG) assay that includes a broad-

spectrum general primers (BSGP5+/6+)-PCR amplifying the L1 region (~150 bp) of 51 mucosal HPV types, as previously described by Schmitt et al. (29). PCR products were detected by hybridization to HPV type-specific probes coupled to fluorescently labelled xMAP Luminex beads (29, 30). Human beta-globin served as internal control and samples that were negative for both HPV and beta-globin were excluded from the analysis (invalid). The E6*I transcripts of the HPV types 16 and 33 (identified in the MPG assay) were assessed by reverse-transcriptase PCR generating short amplicons (65 and 74 bp, respectively) and hybridization as previously described (31). RNA integrity was assessed by co-amplification of ubiquitin C mRNA (85 bp). Samples positive for HPV and/or ubiquitin C were valid.

2.5 Statistical Analysis

Fisher's exact test was applied for testing relationships between categorical variables using the GraphPad Prism 8 software. P values <0.05 were considered statistically significant.

3 RESULTS

3.1 Patients' Demographics

Demographic data are displayed in **Table 1**. The mean age at diagnosis was 61.6 years. There were more male than female patients in our population. Eight (16%) patients had surgery only. Surgery followed by adjuvant radiotherapy/radiochemotherapy was performed in 24 (49%) cases, whereas 17 (35%) patients underwent primary radiotherapy/radiochemotherapy. Follow-up data and data on SPTs are presented in paragraph 3.4. The HPV status of the SPTs was not available for analysis.

3.2 HPV Detection in Tumor Tissue

HPV-positive OPSCC were defined by double positivity for both $p16^{INK4A}$ immunhistochemical staining and HPV DNA by BSGP5+/6+-PCR. In brief, 26 (53%) tumors were attributable to HPV, comprising 22 (85%) tumors driven by HPV16 and four (15%) tumors driven by HPV33. All other tumors were defined as non-HPV-related (**Table 2**).

Forty-nine (100%) samples detached from diagnostic tumor biopsies were available for HPV mRNA analysis. HPV16 or HPV33 mRNA was detected in 23 of 26 (88%) samples of tumors presenting both HPV DNA positivity and $p16^{INK4A}$ overexpression in the diagnostic biopsies or surgical specimens (**Figure 1**). The remaining three (12%) HPV mRNA-negative study samples showed no dysplasia as well as $p16^{INK4A}$ negativity in the histological examination. Finally, no HPV mRNA was found in tumors defined as non-HPV-related.

3.3 Histological Examination, $p16^{INK4A}$ Immunohistochemistry and HPV Detection in Normal Appearing Mucosa Adjacent to and Distant From the Tumor

Forty-eight samples from normal appearing mucosa close to the tumor and 45 ones from normal appearing oropharyngeal tissue distant from the tumor were available for histological examination, $p16^{INK4A}$ immunohistochemistry, HPV DNA as well as HPV mRNA analysis. Matching results are displayed in **Table 3**. Ten samples (5 adjacent ones, 5 distant ones), labeled as invalid, failed the multiplex HPV genotyping assay, since they tested negative for both HPV and human beta-globin. No invalid result was observed for HPV mRNA analysis.

3.3.1 Patients With a Non-HPV-Related Index Tumor

Among the 23 patients with a non-HPV-related index tumor, 19 (83%) samples of normal appearing mucosa adjacent to the tumor showed neither carcinoma nor high-grade dysplasia in the histological examination. Three (13%) adjacent mucosa samples presented invasive carcinoma, most likely manifestations of the primary tumors. One (4%) adjacent mucosa specimen showed high-grade dysplasia. Here, no $p16^{INK4A}$ overexpression was observed, although $p16^{INK4A}$ positivity was described in the index tumor.

In the distant mucosa samples' analysis, carcinoma was detected in only one specimen, later identified as a synchronous second primary tumor of the contralateral tonsil in a patient with a Stage IV, non-HPV-related index tonsillar carcinoma. The remaining distant tissue specimens showed neither carcinoma nor high-grade dysplasia in the histological examination.

None of the adjacent and distant mucosa specimens presented $p16^{INK4A}$ overexpression.

Neither HPV DNA nor mRNA was detected in the adjacent and distant tissue samples of the 23 patients with non-HPV-related index tumors.

3.3.2 Patients With an HPV-Related Index Tumor

Among the 26 patients with an HPV-related index tumor, 25 adjacent mucosa samples and 24 distant ones were available for analysis. Twenty-four (96%) adjacent mucosa samples showed no dysplasia (**Figure 1**); in one (4%) adjacent mucosa specimen carcinoma was detected, also manifesting $p16^{INK4A}$ overexpression. $p16^{INK4A}$ overexpression was not found in dysplasia-free adjacent samples.

Neither dysplasia nor $p16^{INK4A}$ overexpression were identified in distant tissue specimens.

HPV DNA was detected in nine of the 25 (36%) adjacent mucosa samples and in four (17%, 1 HPV DNA-invalid excluded) distant ones (**Figure 2**). HPV genotyping revealed the same HPV type that was present in the index tumor, which was HPV16 in all cases.

HPV16 mRNA was detected in a single (4%), HPV DNA-positive adjacent mucosa sample, which also presented carcinoma and $p16^{INK4A}$ overexpression in the histological examination. Otherwise, all remaining adjacent and distant tissue samples tested negative for HPV16 and HPV33 mRNA.

3.4 Second Primary Tumors and HPV Detection

The median follow-up time of the patient cohort was 58 months (range 0-101 months). Eight (16%) patients developed at least one second primary tumor (SPT) of the upper aerodigestive tract (UADT). The median interval between diagnosis of the index tumor and onset of SPTs was 12 months (range 0-103 months). As previously stated, the HPV status of the secondary primary tumors, particularly data on $p16^{INK4A}$ immunohistochemistry and

TABLE 1 | Selected demographic data and characteristics of study participants.

		No.	%
Total patients		49	100
Age (years)	Range	29-81	
	Median	62	
Gender	Male	38	78
	Female	11	22
Tobacco Smoking (>10 pack years)	Yes	30	61
	No	16	33
	Unknown	3	6
Alcohol use (>3 Units)	Yes	19	39
	No	30	61
Lifetime sexual partners	0-9	23	47
	10-19	11	22
	≥ 20	11	22
	Unknown	4	8
Tumor site	Tonsil	30	61
	Base of tongue	12	24
	Other subsites	7	14
Stage	Stage I/II ($p16^{INK4A}$-negative)	5	10
	Stage I/II ($p16^{INK4A}$-positive)	24	49
	Stage III/IV ($p16^{INK4A}$-negative)	14	29
	Stage III/IV ($p16^{INK4A}$-positive)	6	12
T stage	T1-T2 ($p16^{INK4A}$-negative)	10	20
	T1-T2 ($p16^{INK4A}$-positive)	22	45
	T3-T4 ($p16^{INK4A}$-negative)	9	18
	T3-T4 ($p16^{INK4A}$-positive)	8	16
N stage	Nx/N0 ($p16^{INK4A}$-negative)	9	18
	Nx/N0 ($p16^{INK4A}$-positive)	5	10
	N1-N3 ($p16^{INK4A}$-negative)	10	20
	N1-N3 ($p16^{INK4A}$-positive)	25	51
Follow-up time (months)	Range	0-101	
	Median	58	

HPV genotyping, was not available for analysis. Details on those patients are demonstrated in **Table 4**. Second primary tumors were significantly more common in patients with non-HPV-related index tumors (7/23, 30%) compared to those with HPV-related index tumors (1/26, 4%, p= 0.0210, Fisher's exact test).

3.4.1 Patients With a Non-HPV-Related Index Tumor

Among patients with non-HPV-related index tumors, five (22%) patients developed at least one SPT of the oral cavity. One (4%) patient was diagnosed with a SPT of the hypopharynx, one (4%) with a SPT of the larynx and another one (4%) with a SPT of the esophagus. Two (9%) patients had a SPT of the oropharynx: one patient was diagnosed with an index non-HPV-related carcinoma of the right soft palate and developed a carcinoma of the right base of tongue after 73 months. As previously mentioned, a synchronous second primary tumor of the contralateral tonsil was detected in a patient with an index non-HPV-related tonsillar carcinoma of the left tonsil.

TABLE 2 | HPV status in tumor tissue.

	No. of cases (%)
HPV-related OPSCCs	**26 (53%)**
HPV16 DNA pos. + $p16^{INK4A}$ IHC pos.	22 (45%)
HPV33 DNA pos. + $p16^{INK4A}$ IHC pos.	4 (8%)
HPV-negative OPSCCs	**23 (47%)**
HPV16 DNA pos. + $p16^{INK4A}$ IHC neg.	3 (6%)
HPV DNA neg. + $p16^{INK4A}$ IHC pos.	4 (8%)
HPV DNA neg. + $p16^{INK4A}$ IHC neg.	16 (33%)

OPSCCs, oropharyngeal squamous cell carcinomas; IHC, immunohistochemistry.

3.4.2 Patients With an HPV-Related Index Tumor

Among patients with an HPV-related index OPSCC, only one (4%) patient, who was initially diagnosed with an HPV-related index OPSCC of the right tonsil, developed a metachronous carcinoma of the ipsilateral base of tongue. However, no HPV DNA nor HPV mRNA were detected in the adjacent and distant mucosa samples of this patient. In this small cohort of patients with HPV-related index tumors, we did not find any evidence of link between HPV detection in normal appearing oropharyngeal tissue and development of second primary tumors.

4 DISCUSSION

In the last decades, HPV-related OPSCCs have gained clinical significance, mostly due to their rising incidence and better prognosis compared to HPV-negative counterparts (3, 32). Although the understanding of the clinical and demographical profile of this disease has considerably improved over the years (33), its natural history still remains largely unknown (18). SPTs

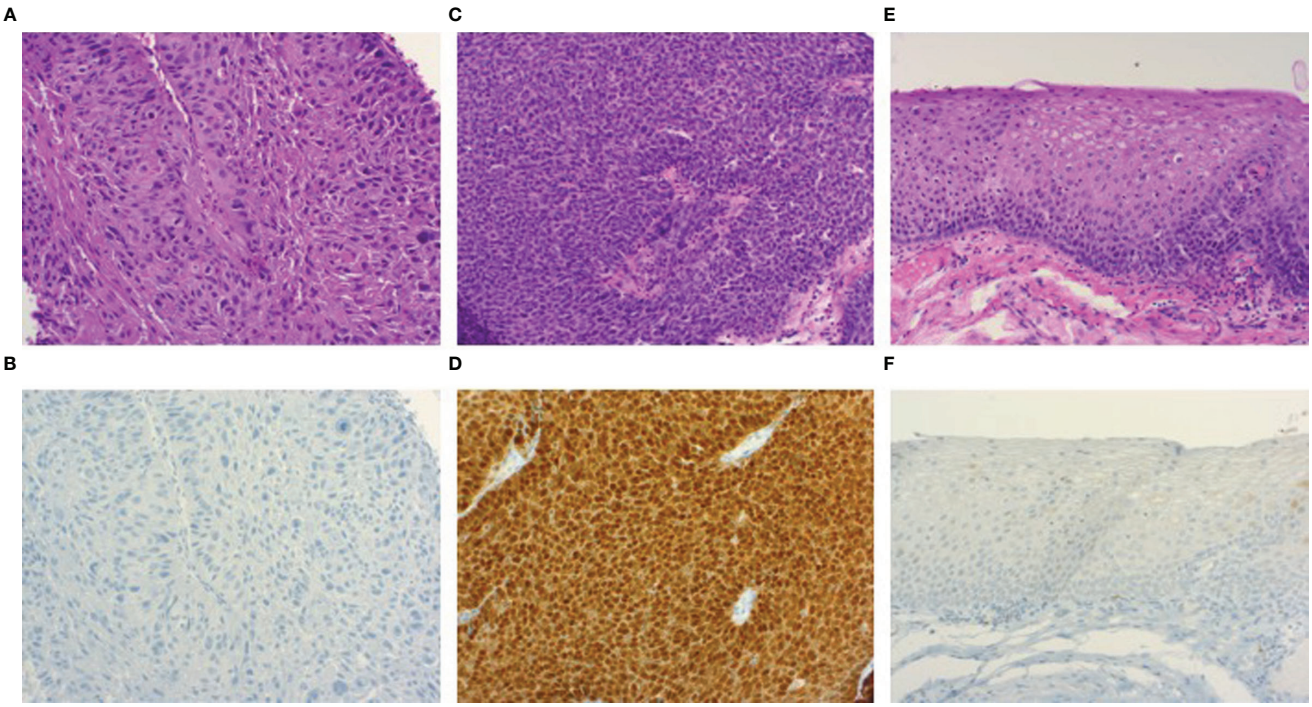

FIGURE 1 | Photomicrographs of hematoxylin/eosin and $p16^{INK4A}$ immunohistochemical stained sections (100x). HPV-negative oropharyngeal squamous cell carcinoma **(A)** showing $p16^{INK4A}$ negativity by immunohistochemistry **(B)**. Image **(C)** demonstrates an HPV-related oropharyngeal squamous cell carcinoma showing $p16^{INK4A}$ overexpression **(D)**, accompanied by a section of dysplasia-free adjacent mucosa **(E)** displaying negativity for $p16^{INK4A}$ **(F)**.

have been increasingly reported in patients with HPV-related OPSCCs, challenging the assumption of a lower risk of SPTs due to the lack of a "tobacco- and alcohol induced field cancerization" (17). It may therefore be hypothesized that detection of high-risk HPV in the oropharyngeal mucosa could indicate areas of «virus-induced field cancerization» and play a role in HPV-related SPT-carcinogenesis. In our study, we prospectively examined the prevalence of a transcriptionally active HR-HPV infection in the normal appearing oropharyngeal tissue in patients with OPSCCs and its potential impact on developing SPTs.

In our cohort, 26 out of 49 (53%) tumors have tested positive for HPV DNA plus $p16^{INK4A}$ positive immunohistochemistry and were defined as HPV-related OPSCCs. This percentage matched current data from Germany (34) as well as data presented by Castellsagué et al. who estimated the HPV-attributable fraction for OPSCCs to be 44.9%-50% in Central-Eastern Europe (35).

Furthermore, we performed testing for HPV E6*I mRNA, since HPV mRNA detection is generally accepted as the gold standard for diagnosis of a transcriptionally active HPV infection (33, 36, 37). High HPV mRNA-detection rates (88%) in samples taken from HPV-related OPSCCs support the reliability of the algorithm comprised of $p16^{INK4A}$ immunohistochemistry plus HPV DNA as surrogate for a transcriptionally active HPV infection in OPSCCs, which was found to have a sensitivity and specificity, respectively, of 96% and 98% (38, 39). In our cohort, however, three samples extracted from HPV-related tumors showed HPV mRNA-negativity as well as lack of high-grade dysplasia or carcinoma in the histological examination and were therefore not representative for OPSCC tissue, suggesting sampling error.

TABLE 3 | HPV detection in normal appearing oropharyngeal tissue adjacent to and distant from the tumor in HPV-positive and negative OPSCCs.

No.	Mucosa adjacent to the tumor				Distant mucosa			
	HPV DNA by BSGP5+/6+ PCR				HPV DNA by BSGP5+/6+ PCR			
HPV mRNA	Positive	Negative	Invalid	Total	Positive	Negative	Invalid	Total
Positive	1	0	0	1	0	0	0	0
Negative	8	34	5	47	4	36	5	45
Total	9	34	5	48	4	36	5	45

BSGP5+/6+ PCR = broad-spectrum general primers 5+/6+-PCR.

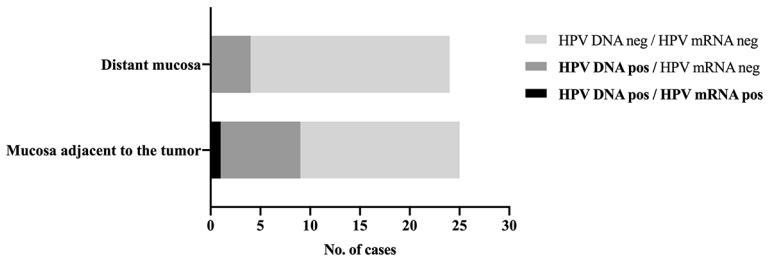

FIGURE 2 | HPV detection in normal appearing mucosa adjacent to and distant from the tumor. Distribution of HPV DNA-positive and HPV mRNA-positive samples in normal appearing tissue adjacent to and distant from the tumor in patients with an HPV-related index oropharyngeal squamous cell carcinoma. HPV16 mRNA was detected in a single $p16^{INK4A}$-positive and HPV DNA-positive adjacent mucosa sample, which also showed carcinoma in the histological examination, likely from the index tumor, suggesting sampling error.

In the mucosa adjacent to the tumor, carcinoma was detected in four specimens, most likely manifestations of the primary tumors. The matching $p16^{INK4A}$ status supports this assumption. One further adjacent mucosa specimen showed high grade dysplasia. Here, no $p16^{INK4A}$ overexpression was detected, while $p16^{INK4A}$ immunopositivity was described in the HPV DNA-negative index tumor, possibly indicating different neighboring dysplastic foci or intratumor heterogeneity, since this patient had history of tobacco and alcohol consumption (18, 40). Moreover, there was no $p16^{INK4A}$ overexpression in the dysplasia-free samples adjacent to and distant from the tumor. Implications of $p16^{INK4A}$ immunohistochemical testing in normal oropharyngeal tissue should be further inquired, since $p16^{INK4A}$ overexpression was already detected in tumor-free tonsil samples (28%) from patients treated for chronic tonsillitis by tonsillectomy (41). Similarly, Begum et al. reported up-regulated $p16^{INK4A}$ in nonneoplastic oropharyngeal epithelium, while HPV DNA was only found in carcinoma and dysplastic epithelium (19), questioning the suitability of $p16^{INK4A}$ as a single marker for definition of an HPV-related OPSCC.

The HPV detection methods used in our study stood out due to their high analytical sensitivity for HPV DNA (100 and 10 plasmid copies of HPV16 and HPV33 detectable, respectively) and E6*I mRNA (10 *in vitro* transcript copies of HPV16 and HPV33 detectable) (29, 31). 10 samples failed the multiplex HPV genotyping assay, most probably reflecting poor DNA quality. No invalid result was observed for HPV mRNA analysis, since this assay was optimized for technically challenging material like archived FFPE samples by the use of ultra-short amplicons.

HR-HPV-presence as by HPV DNA detection was found in normal appearing mucosa samples (36% adjacent and 17% distant samples) of patients with an HPV-related index OPSCC, inserting in a broad landscape of data on the prevalence of oral and oropharyngeal HR-HPV infection.

According to Joseph et al., who demonstrated consensus genetic sequences of HPV16 in synchronous tonsillar carcinomas, it could be hypothesized that the same HPV infection found in tumor tissue also related to the one detected in adjacent and distant mucosal specimens, since genotyping consistently revealed HPV16 (11). Lack of HPV detection in the adjacent and distant mucosa of patients with a non-HPV-related index OPSCC also corroborates this interpretation. However, an incidental second infection with HPV16 should also be taken into consideration. In fact, several studies described a moderate (6.9% - 13.1%) to low (0 – 6.3%) HR-HPV prevalence in respective oral gargles (20, 21, 42) and non-malignant tonsillar tissue (22–26). In line with our results, a further study showed

TABLE 4 | Selected demographic data and characteristics of patients with a second primary tumors.

Sex/age (years)	Follow-up (months)	Index tumor HPV-status	Second primary tumor site	Onset period	Interval months	Tobacco smoking (>10 pack years)
M/54	81	HPV-negative	oral cavity	MC	12	yes
			oral cavity	MC	36	
			oropharynx	MC	73	
M/51	101	HPV-positive[a]	oropharynx	MC	90	yes
			hypopharynx	MC	103	
M/70	95	HPV-negative	oral cavity	MC	7	yes
M/61	41	HPV-negative	esophagus	S	0	yes
			oral cavity	MC	24	
M/56	8	HPV-negative	larynx	S	0	yes
M/45	20	HPV-negative	oropharynx	S	0	yes
M/49	31	HPV-negative	oral cavity	S	0	yes
F/68	51	HPV-negative	hypopharynx	S	0	yes
			oral cavity	MC	22	

F, female; M, male; MC, metachronous; S, synchronous. [a], as defined by double positivity for both $p16^{INK4A}$ immunhistochemical staining and HPV DNA.

HPV DNA detection in 21% of 108 pharyngeal endoscopic biopsies of patients with an index tonsillar carcinoma (43). Nevertheless, reports on HPV prevalence are afflicted by lack of standardized detection techniques and by small sample sizes, making data interpretation very challenging. Moreover, HPV DNA analysis is usually employed as primary HPV detection method in most studies (20–26), even if HPV DNA detection may reveal a transient, rather than a transforming HPV infection (44). A recent systematic review investigated the prevalence of oral HR-HPV infection in 28544 healthy individuals and its potential influence on HPV-attributable fractions (Afs) of OPSCCs and HPV-related OPSCC rates (45). In fact, the authors could not find a significant correlation between the oral HPV prevalence and differences in HPV-Afs or HPV-related OPSCC rates across healthy populations (45).

In order to investigate the HPV transcriptional activity in the normal appearing oropharyngeal mucosa we tested for presence of HPV mRNA. Here, a single adjacent mucosa sample showed HPV16 mRNA-positivity. However, here carcinoma cells, likely from the HPV16-positive index tumor, were revealed in the histological examination, suggesting sampling error because of a larger microscopic tumor extension. Furthermore, no HPV mRNA was detected in distant tissue samples. Summarily, no evidence was found for a multifocal transcriptionally active HPV infection in tumor-free oropharyngeal tissue adjacent to and/or distant from the index tumor, corroborating and broadening previous data by Rietbergen et al., who could not find any HPV16 mRNA in tumor-free resection margins of HPV-driven OPSCCs treated by surgery (46).

In this context, the emergent number of reports about second primary tumors (SPT) in patients with HPV-related OPSCC becomes increasingly relevant, since a link between a potential multifocal HPV infection and SPTs-development has been hypothesized (11–17, 47). Indeed, as cited above, Joseph et al. detected the same HPV16 variant by E6 DNA sequencing in four pairs of HPV tonsil carcinomas, suggesting HPV multifocality (11). This evidence is challenging previous studies as well as our observations, which indicated a lower risk of development of SPT in patients with HPV-related tumors in contrast to HPV-negative OPSCCs patients, especially in never smokers (5, 7). Now, in the extensive systematic review presented by Strober et al. the prevalence of SPTs in HPV-related OPSCC patients ranged from 0.95% to 10% and the contralateral tonsil was the most common site for development of a HPV-mediated SPTs (17). Whereby, we wondered if HPV detection in tumor-free neighboring tissue and in the contralateral tonsil might correlate with the development of second primary tumors (SPT) and indicate a possible «virus-induced field effect». In the median follow-up time of 58 months, only one patient who was initially diagnosed with an HPV-related index OPSCC and had persistent smoking habits, developed SPTs during follow-up. Neither HPV mRNA nor HPV DNA could be detected in the adjacent or distant tissue biopsies of this patient. In this small cohort, we found no evidence of link between HPV detection in normal appearing mucosa and origin of second primary tumors. Unfortunately, no information about the HPV status of the SPTs ($p16^{INK4A}$ immunohistochemistry and HPV genotyping) was available for analysis, which represents a clear limitation of this study. Moreover, the interpretation of these data is limited by a small number of participants and limited follow-up documentation. Further, only FFPE tissue samples were available for DNA and mRNA isolation, which are naturally inferior in quality compared to fresh frozen material. Since HPV-related OPSCCs are usually small and difficult to detect (48), challenging samples' acquirement and therefore possible sampling error should also be taken into consideration. Larger cohort studies including fresh frozen material are clearly needed in order to validate this data.

In conclusion, we present a prospective cohort study addressing the prevalence of HR-HPV infection in the normal appearing oropharyngeal tissue of OPSCC-patients with a robust HPV detection methodology. We showed that HPV was detectable but not transcriptionally active in adjacent/distant tumor-free oropharyngeal tissue. In this small cohort, we found no association between HPV detection and development of SPTs. These data suggest that a multifocal transcriptionally active HPV infection, hinting a «virus-induced field cancerization», may not be pertaining to HPV-related OPSCC. Future investigations on the mechanism underlying the development of HPV-related SPTs are urgently needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee Canton St. Gallen. The patients/participants provided their written informed consent to participate in this.

AUTHOR CONTRIBUTIONS

VG contributed to data acquisition, data analysis and interpretation, manuscript writing. LS contributed to data acquisition, data analysis and manuscript editing. MB contributed to study conception and design, data interpretation and overall review. KI, NR, and DH contributed to data acquisition and analysis. SS and MP contributed to study conception and design. WJ contributed to acquisition of data. All authors contributed to the article and approved the submitted version.

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