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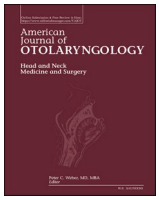

The inflammatory microenvironment in patients with advanced and relapsing hypopharyngeal squamous cell carcinoma

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ABSTRACT

Hypopharyngeal squamous cell carcinoma (HPSCC) is frequently diagnosed at an advanced stage and is associated with limited therapeutic options and poor prognosis. Increasing evidence highlights the relevance of the tumour microenvironment (TME), particularly inflammatory cell populations, for tumour progression and treatment response. Tumour-associated macrophages (TAMs), especially CD68-positive subsets, have emerged as potential diagnostic and prognostic markers. Factor X (FX), a key component of the coagulation cascade with additional immunomodulatory functions, may contribute to macrophage recruitment and influence TME composition. This study aimed to characterise the inflammatory landscape of advanced HPSCC with a focus on FX_CD68-positive TAMs and to explore associations with clinical outcome.

Histopathological and immunohistochemical analyses were performed on tumour samples from 31 patients with advanced HPSCC. Acute inflammation was graded based on neutrophil infiltration, while chronic inflammation was assessed through lymphoid aggregates and overall inflammatory cell density. FX_CD68-positive macrophages were quantified and correlated with clinical data including stage, treatment modality, and relapse.

All tumours demonstrated pronounced acute and chronic inflammation. Most patients ($n = 29$) presented with UICC stage II–IV disease. Eleven patients (35%) developed a relapse, typically showing stronger chronic inflammation in the primary tumour. Recurrent tumours displayed heterogeneous densities of FX_CD68-positive TAMs, whereas specimens obtained after radio(chemo)therapy exhibited reduced inflammatory infiltration.

These findings indicate that HPSCC is a highly immunogenic tumour entity. Components of the TME, particularly FX_CD68-positive TAMs, may represent promising biomarkers for risk stratification and could support the development of personalised therapeutic strategies.

1. Introduction

Head and neck squamous cell carcinomas (HNSCC) are a heterogeneous group of malignancies that include cancers of the oral cavity, oropharynx, larynx, nasopharynx, and hypopharynx, together accounting for about 5% of all newly diagnosed cancers worldwide and 4% of cancer-related deaths [1,2]. Hypopharyngeal squamous cell carcinoma (HPSCC) represents 5–10% of HNSCC cases and is strongly associated with tobacco use and alcohol abuse, while only a minority of cases are related to human papillomavirus (HPV) infection [2,3]. Unlike HPV-positive oropharyngeal carcinoma, which responds favourably to chemoradiotherapy (CRT) and carries a better prognosis [4], HPV status in HPSCC does not appear to confer a prognostic benefit.

HPSCC is typically diagnosed at an advanced stage, with a 5-year overall survival rate of around 40% [2]. Treatment usually involves multimodal therapy, including surgery followed by adjuvant (chemo) radiotherapy or primary CRT [5]. However, hypopharyngeal surgery is often invasive and functionally disabling, leading to greater use of organ-preserving CRT strategies. Recent studies, however, suggest that primary surgery followed by adjuvant therapy may achieve superior

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survival outcomes compared with definitive CRT [6,7] Identifying prognostic biomarkers that can guide therapy decisions and individualise treatment therefore remains an important clinical need.

Systemic inflammatory markers such as the systemic inflammation response index have recently been reported as prognostic in HPSCC [8]. Yet, tumour-associated inflammation is increasingly recognised as a hallmark of cancer, and the tumour microenvironment (TME) may provide more direct prognostic insights than peripheral blood markers [9–11]. The TME comprises a complex network of immune cells, including T cells, neutrophils, and tumour-associated macrophages (TAMs), all of which influence tumour progression and response to therapy [12,13]. Immune checkpoint inhibitors (ICI) have become a first-line option in recurrent or metastatic HNSCC but their benefit in combination with CRT in the curative setting remains unproven underlining the importance of better understanding immune–tumour interactions [14].

TAMs are of particular interest because of their role in tumour growth, angiogenesis, and immune evasion [15,16]. CD68 is widely used as a macrophage marker, and high CD68 expression has been associated with poor outcomes across several tumour types, including glioblastoma [17,18]. Factor X (FX), best known for its role in the coagulation cascade, also participates in non-coagulant signalling pathways relevant to immune regulation and cancer [19,20]. Recent studies suggest a functional link between FX expression and TAM recruitment raising the possibility that FX-positive TAMs may influence tumour biology and prognosis [21].

Given that HPV is not an established biomarker in HPSCC and that no routine inflammatory profiling is currently in place, there is a pressing need for novel markers to support prognosis and treatment stratification. The aim of this study was therefore to characterise the TME in advanced HPSCC, focusing on neutrophilic infiltration, the presence of acute and chronic inflammation, and the role of FX_CD68-positive TAMs in relation to patient outcomes.

2. Methods

2.1. Clinical assessment

We conducted a retrospective analysis of consecutive patients diagnosed with hypopharyngeal squamous cell carcinoma (HPSCC) at our centre over a 36-month period (January 2020 to December 2022). Medical records were reviewed by experienced clinicians (RS, HG) to extract all relevant clinical data. Patients were included based on the following criteria:

Inclusion criteria:

Any patients with a Diagnosis of HPSCC, that were treated at our centre during the study period and presented with either locally advanced disease (T2–T4) or relapsing disease.

Exclusion criteria:

Patients with T1-stage tumours without evidence of relapse or metastatic disease at the time of analysis.

2.2. Pathology

Histopathological samples were evaluated using a standardised, clinically applicable scoring system based on haematoxylin and eosin (H&E) staining, focusing on the extent of chronic and acute inflammation in the tumour microenvironment (TME) (compare Fig. 1).

Chronic inflammation was semi-quantitatively scored as:

- Absent (score = 0)
- Mild (score = 1)
- Moderate (score = 2)
- Severe (score = 3)

This scoring was based on the density of inflammatory cells (lymphocytes, plasma cells, macrophages) and the presence of lymphoid follicle formation.

Acute (active) inflammation was scored as:

- Absent (score = 0)
- Mild (score = 1)
- Moderate to severe (score = 2)

Scoring was based on the density of neutrophilic granulocyte infiltration in the TME [28].

2.3. Immunohistochemistry

For the analysis of FX_CD68-positive macrophages, five high-power

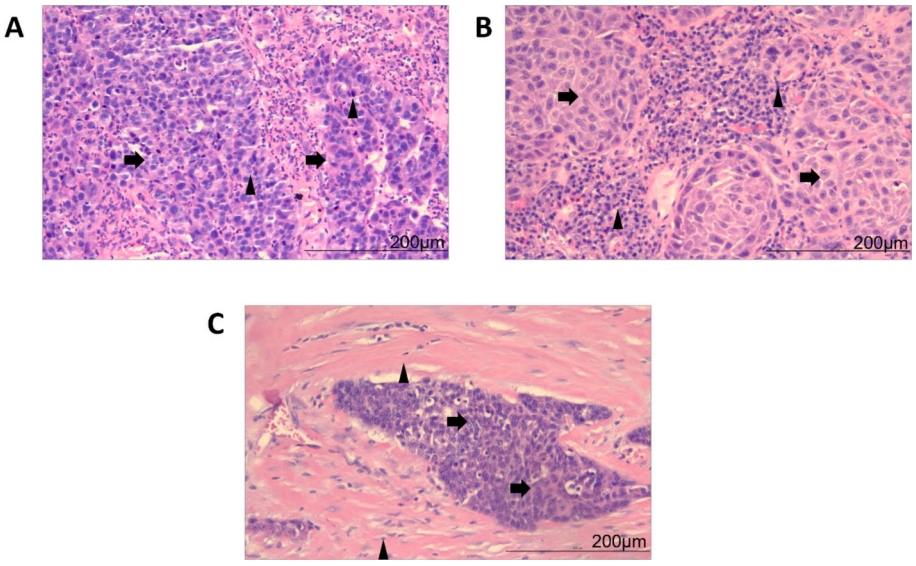

Fig. 1. Representative histological sections of HPSCC illustrating varying degrees of tumour-associated inflammation: A: Squamous cell carcinoma with dense infiltration of neutrophils, indicating pronounced active inflammation. B: Squamous cell carcinoma with dense infiltrates of lymphocytes, macrophages, and plasma cells, along with scattered neutrophils, characteristic of chronic inflammation. C: Squamous cell carcinoma with only sparse inflammatory cells (Arrow: tumour cells; Arrowhead: inflammatory cells; scale bar: 200 µm) (HPSCC – hypopharyngeal squamous cell carcinoma).

fields (HPFs) per histopathological sample were evaluated (compare Fig. 2). The average count was normalized to square millimetres of tumour tissue. For all patients with primary disease, samples from the time of initial diagnosis were analysed. In three patients, only samples from recurrent or metastatic disease were available. Additionally, seven more samples were obtained from patients who developed relapse or metastases after the initial diagnosis. In one patient, a third sample from a distant metastasis was also included.

2.4. Statistical analysis

A p-value of <0.05 was considered statistically significant. Comparisons between independent groups were performed using unpaired t-tests or one-way analysis of variance (ANOVA), as appropriate. All statistical analyses were conducted using GraphPad Prism (Version 6).

2.5. Ethical approvement

In this study, only health data that is collected in the clinical routine was analysed retrospectively. So-called "third parties" did not have access to the data and publication occurs exclusively in anonymized form. The Ethics Committee of the local Medical Association clearly states that patient informed consent can be waved in this kind of studies and furthermore refrains from providing advice in such cases, citing the State Hospital Act (§36 and §37).

3. Results

Thirty-one consecutive patients with UICC (Union for International Cancer Control) stage I–IV HPSCC were included (age range: 52–83 years; 5 female, 26 male). Twenty-nine patients presented with advanced-stage (II–IV) disease at initial diagnosis. One patient was diagnosed at stage II; 23% (n = 7) had stage III disease, 35% (n = 11) had stage IVA, and 26% (n = 8) had stage IVB disease. Two patients initially diagnosed with stage I later developed fulminant (rcT4) local relapse. Additionally, two patients presented with metastatic stage IVC disease at first diagnosis. Lymph node metastases were present in 77% of patients at initial presentation.

Eleven patients (35%) developed recurrent or metastatic disease. Three of these exhibited both lymph node recurrence and either local relapse or distant metastases. Four patients developed distant metastases, involving either bone or lung (s. Table 1). Four patients had nodal recurrence, and six experienced local tumour relapse. One patient developed second and third metachronous carcinomas of the oropharynx. Time to relapse ranged from 5 to 60 months.

Only one patient showed focal p16-expression in a lymph node metastasis. As HPV-positive HPSCC is rare, HPV testing was not performed in 11 patients. Among the 25 patients with available data on smoking habits, all were heavy smokers (≥20 pack-years). Four patients reported no alcohol consumption; in six, alcohol data were unavailable. The remaining 21 consumed alcohol regularly, with several reporting past or ongoing abuse.

Thirteen patients (42%) received primary chemoradiotherapy (CRT), and thirteen (42%) underwent primary surgery, with seven of these receiving adjuvant radiotherapy. Five patients received palliative treatment, including best supportive care or palliative radiotherapy (Quad Shot).

Histological evaluation of primary (and, where available, relapsing) tumours revealed absent active inflammation in 10% (vs. 11% in relapsed samples), mild in 39% (vs. 44%), and moderate to severe in 50% (vs. 44%). Chronic inflammatory infiltration was mild in 25% of primary tumours (vs. 67% in relapsed) and moderate to severe in 75% (vs. 33%) (s. Fig. 3). All eleven patients who experienced relapse initially showed moderate or severe chronic inflammation; however, their recurrent tumours demonstrated a shift to predominantly mild inflammation. Active inflammation in this subgroup was moderate to severe in most patients at diagnosis but tended to be reduced at relapse. One case was showing absent inflammation, four mild, and four moderate to severe inflammation scores. Among six patients who underwent primary or adjuvant radiotherapy and later relapsed, inflammation scores were markedly decreased in post-treatment specimens collected at relapse.

The density of FX_CD68-positive macrophages ranged from 1.7 to 30 cells/mm^2 (s. Fig. 4). Among patients who later relapsed, FX_CD68 levels at first diagnosis varied from 0.4 to 3.6 cells/mm^2 (s. Figs. 4 and 5). No significant correlation was observed between FX_CD68 density and tumour stage. In patients with stage IVC disease at first diagnosis, moderately increased densities were recorded (2.5 and 5.8 cells/mm^2). In four relapsed tumours the measured FX_CD68 expression was lower, compared to the expression in primary tumour. Only two patients with relapsing disease showed FX_CD68 levels below 4 cells/mm^2 in their recurrent tumours (Patients 13 and 26, s. Fig. 5) both of whom had received primary surgery followed by adjuvant radiotherapy. In two other patients, both treated with total laryngectomy, neck dissection,

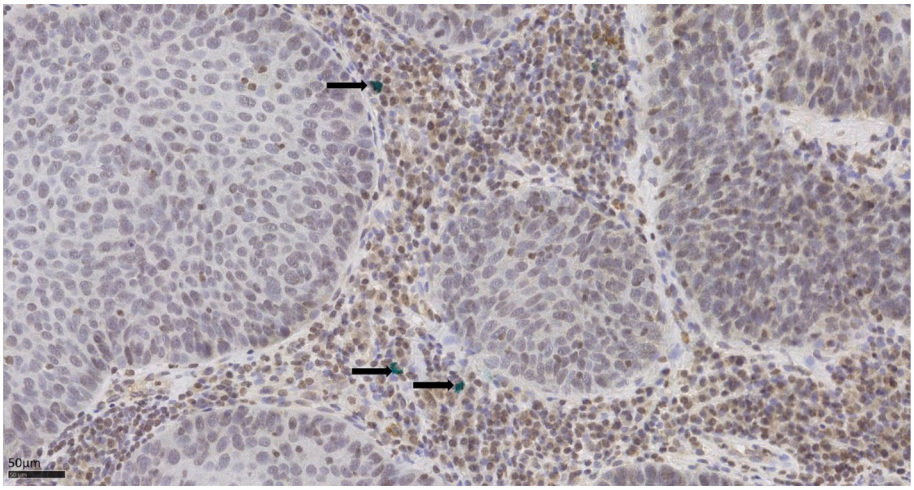

Fig. 2. Representative histological section showing FX_CD68 staining in HPSCC. Hypopharyngeal squamous cell carcinoma cells with surrounding FX- and CD68-positive macrophages (Asterisk: cancer cells; Arrow: double positive macrophages expressing CD68 (green) and FX (brown); Scale bar: 50 µm) (FX – Factor X; HPSCC – hypopharyngeal squamous cell carcinoma).

Table 1
Epidemiological and clinical data of the 31 patients of this cohort.

#	TNM at time of first diagnosis	Sex	Age at first diagnosis	Relapse/metastatic disease (time to relapse after first diagnosis)	Therapy at first diagnosis
1	cT4 cN1 cM0	m	73	–	Planned primary CRT, Patient wished therapy at another centre close to home, no further contact
2	pT4a pN3b cM0	m	69	Local relapse (38 months)	Surgery, followed by adjuvant radiation
3	cT3 cN0 cM0	f	77	–	Best supportive care
4	pT3 pN1 cM0	m	64	–	Surgery, adjuvant radiation
5	cT2 cN0 cM0	m	52	–	Primary CRT
6	pT4a pN0 cM0	m	66	Second and third carcinoma (both oropharyngeal; 19 and 36 months)	Surgery
7	cT4b cN1 cM0 p16-	m	82	–	Best supportive care
8	cT4 cN2c cM0 p16-	m	55	Local relapse (10 months)	Primary radiation
9	pT2 pN1 cM0	m	79	–	Surgery, adjuvant chemoradiotherapy
10	cT3 cN2c cM0	m	71	–	Primary CRT
11	cT3 cN2b cM0	m	53	–	Primary CRT
12	cT3 cN2bcM0	m	67	–	Primary CRT
13	pT4a pN3b cM0	f	64	Relapse with distant metastasis (29 months)	Surgery, adjuvant CRT
14	pT3 pN3b cM0	m	75	–	Surgery
15	cT3 cN0 cM0	m	58	–	Primary CRT
16	cT4a cN2b cM0	m	72	–	Planned Primary CRT, after diagnosis no further contact
17	cT2 cN3b cM0	m	68	–	Surgery, adjuvant CRT
18	cT4a N2b cM1 (PUL, HEP, OS)	f	64	–	Best supportive care
19	cT3 cN1 cM0	m	66	–	Primary CRT
20	cT4b cN2b cM0	m	56	–	Palliative radiation
21	cT2 cN2b cM0	m	83	Lymph nodes residuum; distant metastasis and relapse (5 months; respective 10 months)	Primary CRT, Salvage Neck Dissection left side
22	cT4b cN0 cM0	m	57	Distant metastasis (18 months)	Primary CRT
23	cT2 cN2a cM0	m	74	–	Surgery, adjuvant CRT
24	pT4a pN0 cM0	m	60	Lymph node relapse (6 months)	Surgery
25	cT3 cN0 cM0	m	67	Lymph node relapse; Local relapse (6 months; respective 13 months)	Primary CRT, later Salvage-ND, Salvage-Laryngectomy
26	cT4a cN2b cM0	f	62	Distant metastasis (5 months)	Primary CRT
27	pT2 pN2a cM0	m	72	–	Surgery, adjuvant CRT
28	cT4 cN2c cM1 (PUL)	m	59	–	Best supportive care (Patient declined therapy, planned CRT)
29	pT4a pN0 cM0	m	67	Local relapse & lymph node relapse (11 months); recurrent lymph node relapse (38 months)	Surgery
30	pT1 pN0 cM0	m	54	Local relapse (36 months)	Surgery, adjuvant RT
31	pT1 pN0 cM0	f	73	Local relapse (60 months)	Surgery

and adjuvant CRT, FX_CD68 levels were notably reduced at relapse—from 15 to 7.5 cells/mm^2, and from 10 to 2.5 cells/mm^2, respectively (Patients 2 and 13, s. Fig. 5).

4. Discussion

In this study, we demonstrate that hypopharyngeal squamous cell carcinoma (HPSCC) is characterised by a highly immunogenic tumour microenvironment (TME) with frequent moderate to severe inflammatory infiltration. Both acute and chronic inflammatory responses were prevalent at diagnosis, and patients who later relapsed typically exhibited stronger chronic inflammation in their primary tumours. At relapse, however, inflammatory infiltration was markedly reduced, particularly in patients previously treated with radiotherapy (RT) or chemoradiotherapy (CRT). Furthermore, FX_CD68-positive tumour-associated macrophages (TAMs) displayed variable densities, with some patients showing reduced levels following RT, while others maintained or even increased expression.

Chronic inflammation is a well-recognised driver of carcinogenesis and has been linked to risk factors such as tobacco and asbestos exposure [3,22]. In our cohort, virtually all patients were heavy smokers, and many reported alcohol consumption or abuse. Patients with ongoing or past tobacco use showed higher levels of chronic inflammation, suggesting a lasting effect of smoking on the TME. In contrast, no clear relationship was observed between alcohol consumption and acute inflammation. These findings support the established role of chronic exposure to carcinogens in shaping the immune landscape of HPSCC.

TAMs represent a central component of the TME and are known to promote tumour growth, angiogenesis, and immune evasion [14]. Immune checkpoint inhibitors have emerged as first-line therapy in recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) [23]. In other head and neck cancer subgroups, as for example laryngeal cancer, high TAM density and PD-L1 expression have been associated with poor prognosis [24]. Our results are in line with these findings, showing increased densities of FX_CD68-positive macrophages in metastatic disease and variable levels at relapse. Importantly, RT appears to remodel the TME, reducing inflammatory infiltration in our series and altering macrophage density. This observation is consistent with prior reports describing immunomodulatory effects of RT, including recruitment and functional changes of macrophages.

The role of Factor X (FX) in cancer biology is increasingly recognised. Beyond its role in coagulation, FX influences signalling cascades and has been shown to drive TAM recruitment in glioblastoma. The prognostic role of CD68-positive macrophages varies across tumour entities. In Hodgkin lymphoma and advanced thyroid cancer, high densities correlate with poorer survival, while in nasopharyngeal carcinoma, they are associated with improved disease-free survival [25–27]. In our cohort, FX_CD68-positive macrophages were moderately elevated in metastatic cases at diagnosis, supporting a possible role for FX in shaping the HPSCC immune landscape. While survival associations could not be determined due to limited follow-up, patients who died during observation showed both moderate and high FX_CD68 densities, suggesting that further studies are warranted.

Clinically, our findings highlight the importance of the TME in HPSCC, a subgroup of head and neck squamous cell carcinoma (HNSCC) that remains understudied due to its relative rarity. Unlike oropharyngeal SCC, where HPV status is routinely tested and guides therapy, biomarker-driven approaches for HPSCC are lacking. Our data suggest

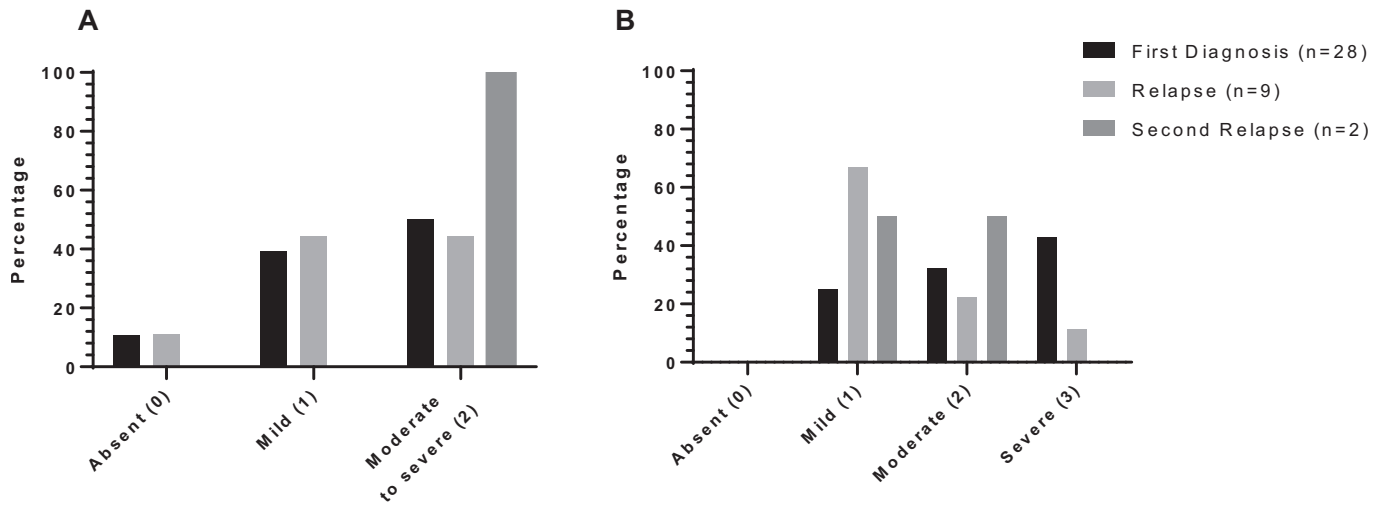

Fig. 3. Distribution of active and chronic inflammation at first diagnosis and relapse. A: Active inflammation, scores as absent, mild or moderate to severe. B: Chronic inflammation scored as absent, mild, moderate or severe. Black bars represent samples at first diagnosis (n = 29), light grey bars samples at first relapse (n = 9), and dark grey bars samples at second relapse (n = 2). No statistically significant differences were observed.

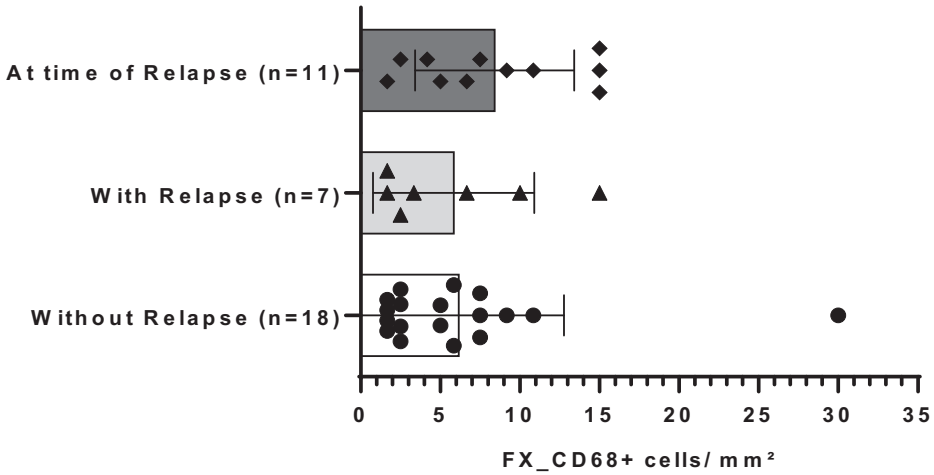

Fig. 4. Comparison of FX_CD68-positive cell density (cells/mm^2) in relapsing disease. The bottom two bars represent samples taken at first diagnosis, divided into patients without a reported relapse (bottom bar) and those who later developed relapse or metastatic disease (middle bar). The top bar shows samples from relapsing tumours. Note: Pathology specimens from the time of first diagnosis were not available for all patients in the relapse cohort. No statistically significant differences were observed. (FX – Factor X).

that inflammatory signatures, including TAM and FX-related markers, may provide prognostic insights and could help stratify patients for immunotherapy or TAM-targeted therapies, which are currently in clinical testing [28,29].

Smoking and alcohol remain the primary risk factors for HPSCC. Human papillomavirus (HPV) is rarely implicated, with reported prevalence ranging from 4 to 20% [30,31]. In our cohort, only one tumour tested positive for HPV. The prognostic significance of HPV in HPSCC remains debated; some studies suggest improved survival, while others do not association [3,30,31]. Unlike oropharyngeal squamous cell carcinoma (OSCC), where p16 immunohistochemistry is routinely used to assess HPV status and guide therapy, no standardised biomarker-based diagnostics exist for HPSCC. Recent OSCC trials, such as Lee et al. and De-ESCALaTE, have explored therapy de-escalation in HPV-positive patients, with mixed results [23,30,32–35]. While some protocols (e.g. reduced-dose RT based on tumour hypoxia) showed comparable outcomes, others (e.g. RT + cetuximab vs RT + cisplatin) resulted in worse survival and recurrence rates [33,34,36].

Although RT and chemotherapy may induce tumoural inflammation, six patients in our cohort who received RT at initial diagnosis showed reduced chronic and acute inflammation at relapse. Similarly, FX_CD68 expression was decreased in some and increased in others following treatment, highlighting the complex immunomodulatory effects of RT. Emerging evidence suggests that specific immune signatures, such as HLA class I expression and CD68 density, may predict survival or therapy response in HPV-negative HPSCC [37,38]. Despite their potential, such biomarkers are not yet incorporated into routine diagnostics. Unlike OSCC, HPSCC lacks standardised, biomarker-guided clinical decision-making tools. Assessing inflammation within the TME—using simple, reproducible scoring systems—may provide valuable prognostic insights and inform personalised treatment strategies.

This study has limitations, including its retrospective design, relatively small cohort, incomplete HPV testing, and short follow-up. Nonetheless, we provide novel evidence that both inflammation and FX_CD68-positive TAMs are key features of the HPSCC TME. In summary, HPSCC is characterised by strong immune infiltration at diagnosis, with marked alterations following RT and in relapsed disease. FX_CD68-positive TAMs may contribute to tumour progression and immune modulation, underscoring the potential of TME markers as prognostic tools and therapeutic targets. Future prospective studies in

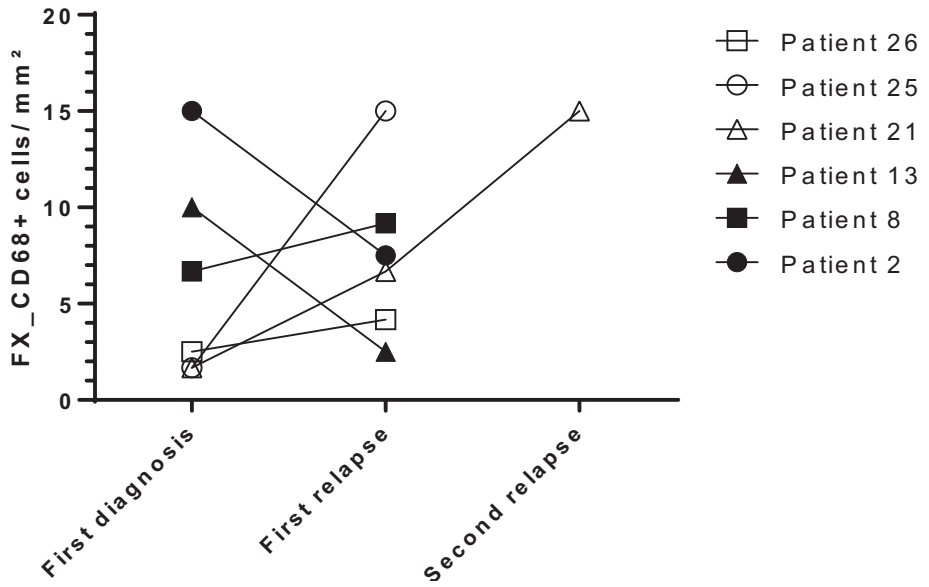

Fig. 5. Longitudinal analysis of FX_CD68-positive cell density from first diagnosis to relapse. For six patients who experienced relapse and for whom tissue samples were available at both first diagnosis and at least one relapse, the number of FX_CD68-positive cells per mm^2 is displayed. Data points connected by lines represent individual patients. The leftmost point for each line indicates the value at first diagnosis, the middle point at first relapse, and the rightmost point (available only for patient 21) at second relapse.
No statistically significant differences were observed. (FX – Factor X).

larger cohorts are needed to validate these findings and to better define the role of TAMs and FX in HPSCC biology.

5. Conclusion

HPSCC is characterised by a highly immunogenic tumour microenvironment with frequent acute and chronic inflammatory infiltration at diagnosis. Patients who later relapsed showed stronger chronic inflammation in their primary tumours but reduced immune infiltration in relapse specimens, particularly following radiotherapy or chemoradiotherapy. FX_CD68-positive macrophages were variably expressed, with moderate elevations in metastatic cases and changes following treatment, suggesting a role in tumour progression and immune modulation.

These findings highlight the potential of inflammatory signatures and TAM subsets, including FX_CD68-positive macrophages, as prognostic markers and therapeutic targets in HPSCC. Prospective studies in larger cohorts are warranted to validate their predictive value and to explore their utility in guiding personalised therapy strategies.

CRediT authorship contribution statement

Anna-Rebekka Staufenberg: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Matthias M. Gaida:** Conceptualization, Investigation, Resources, Supervision, Writing – review & editing. **Claudine Graf:** Resources, Writing – review & editing. **Justus Kaufmann:** Resources, Writing – review & editing. **Christoph Matthias:** Supervision, Writing – review & editing. **Haralampos Gouveris:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

Consent to participate

See Ethical approval.

Consent for publication

See Ethical approval.

Ethical approval

In this study, only health data that is collected in the clinical routine was analysed retrospectively. So-called “third parties” did not have access to the data and publication occurs exclusively in anonymized form. The Ethics Committee of the local Medical Association clearly states that patient informed consent can be waved in this kind of studies and furthermore refrains from providing advice in such cases, citing the State Hospital Act (§36 and §37).

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Data availability

The raw data may be available from the corresponding author upon reasonable request. Each request will be handled according to the local data protection policies.

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