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Original contribution

A novel immunohistochemical scoring system reveals associations of C-terminal MET, ectodomain shedding, and loss of E-cadherin with poor prognosis in oral squamous cell carcinoma☆,☆☆

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Abbreviations: CDx, companion diagnostics; DFS, disease-free survival; ECD, ectodomain; EMT, epithelial-to-mesenchymal transition; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; HNSCC, head and neck squamous cell carcinoma; KM, Kaplan-Meier; OSCC, oral squamous cell carcinoma; OS, overall survival; ROC, receiver operating characteristic; RTK, receptor tyrosine kinase; SCC, squamous cell carcinoma; TMA, tissue microarray; TM, transmembranous; WTS, whole-tissue section.

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Abstract Using tissue microarrays, it was shown that membranous C-terminal MET immunoreactivity and ectodomain (ECD) shedding are associated with poor prognosis in oral cancer. Seen the potential diagnostic value, extrapolation of these results to whole-tissue sections was investigated. Because MET orchestrates epithelial-to-mesenchymal transition (EMT), the results were benchmarked to loss of E-cadherin, a readout for EMT known to be associated with poor prognosis. C-terminal MET, N-terminal MET, and E-cadherin immunoreactivities were examined on formalin-fixed paraffin-embedded parallel sections of 203 oral cancers using antibody clones D1C2, A2H2-3, and NCH-38. Interantibody and intra-antibody relations were examined using a novel scoring system, nonparametric distribution, and median tests. Survival analyses were used to examine the prognostic value of the observed immunoreactivities. Assessment of the three clones revealed MET protein status (no, decoy, transmembranous C-terminal positive), ECD shedding, and EMT. For C-terminal MET−positive cancers, D1C2 immunoreactivity is independently associated with poor overall survival (hazard ratio [HR] = 2.40; 95% confidence interval [CI] = 1.25 to 4.61; and $P = 0.008$) and disease-free survival (HR = 1.83; 95% CI = 1.07−3.14; $P = 0.027$). For both survival measures, this is also the case for ECD shedding (43.4%, with HR = 2.30; 95% CI = 1.38 to 3.83; and $P = 0.001$ versus HR = 1.87; 95% CI = 1.19−2.92; $P = 0.006$) and loss of E-cadherin (55.3%, with HR = 2.21; 95% CI = 1.30 to 3.77; and $P = 0.004$ versus HR = 1.90; 95% CI = 1.20−3.01; $P = 0.007$). The developed scoring system accounts for MET protein status, ECD shedding, and EMT and is prognostically informative. These findings may contribute to development of companion diagnostics for MET-based targeted therapy.

1. Introduction

Approximately 30% of head and neck squamous cell carcinomas (HNSCCs) originate in the oral cavity [1]. Depending on the disease stage and histopathological features, treatment of oral squamous cell carcinoma (OSCC) consists of single-modality surgery, radiotherapy, or a combination of both with or without adjuvant systemic therapy (chemotherapy and/or targeted therapies) [1−4]. Further improvement using more aggressive (chemo) radiotherapy might be outweighed by increased toxicity [5]. Therefore, interest exists to implement targeted therapies in the management of these cancers.

An interesting, yet elusive, target for therapy is the receptor tyrosine kinase (RTK) MET [5−7]. This transmembranous (TM) protein facilitates invasive growth by orchestrating a program similar to epithelial-to-mesenchymal transition (EMT) and is recognized as a negative prognostic factor for HNSCC [8,9]. During EMT, epithelial cells obtain a mesenchymal phenotype by downregulation of epithelial proteins (such as E-cadherin), induction of mesenchymal proteins, and invasion of the extracellular matrix [10,11]. Unfortunately, research into therapies directed against MET has not yet resulted in major survival benefits [12−14]. This might be due to a lack of companion diagnostics (CDx), for which development is challenging for several reasons [9,12,13,15]. Some are of technical nature, ie, absence of specific antibodies and reliable evaluation of immunohistochemistry. Others are related to biology, ie, MET processing and specifically its degradation.

MET can be subjective to presenilin-regulated intramembrane proteolysis. This process encompasses initial cleavage by membrane metalloproteases resulting in shedding of the ectodomain (ECD) from the membrane and subsequent cleavage of the remaining membrane-anchored C-terminal fragment by the γ-secretase complex [16]. Theoretically, proteolytic processing of MET results in four different states of the receptor with respect to the cell membrane: no receptor (no MET), a membrane-anchored N-terminal fragment without the catalytic domain (decoy MET), the complete receptor (MET), and a TM C-terminal fragment with the catalytic domain (TM C-terminal MET). MET processing has necessitated novel approaches to categorize MET immunoreactivity.

Using C- and N-terminal MET antibodies and a tissue microarray (TMA), it was established that C-terminal MET immunoreactivity either is homogeneous (uniform negative or positive staining) across oral and human papillomavirus (HPV)-negative oropharyngeal squamous cell carcinoma or differs between these cancers' center and periphery (variable staining) [17]. It was also shown that MET ECD shedding occurs in OSCC [18]. Both C-terminal MET uniform staining and ECD shedding were found to be associated with poor patient prognosis [17,18]. Seen the potential diagnostic value of these findings, the goal of this study is to investigate the feasibility of extrapolating the TMA results to whole-tissue sections (WTSs). Therefore, a

novel scoring system was developed that addresses tumor heterogeneity by discriminating between immunoreactivities observed in the center and periphery of cancer fields. It was also examined if the scoring system is informative with respect to biological processes such as MET ECD shedding and EMT and whether it is prognostically informative.

2. Materials and methods

2.1. Ethics statement

Human tissues and patient data were used as per *The Code of Conduct for Responsible Use* and *The Code of Conduct for the Used of Data in Health Research* as stated by the Federation of Dutch Medical Scientific Societies [19].

2.2. Patient tissues

Formalin-fixed paraffin-embedded tissue blocks representative for 203 primary OSCCs—surgically removed between 1984 and 2010—were retrieved from the tissue bank of the Department of Pathology of the Leiden University Medical Center. Histopathological characteristics were retrieved from the pathology reports and annotated as per the 7th edition of the Cancer Staging Manual [20]. Using a microtome, 3-µm-thick WTSs were cut in view of immunohistochemical analyses.

2.3. Antibodies and immunohistochemistry

D1C2 (Cell Signaling Technology®; Danvers, MA, USA) detected C-terminal MET as described in the study by De Herdt et al. [17]. A2H2-3 (Eli Lilly and Company; Indianapolis, USA) detected N-terminal MET as described in the study by De Herdt et al. [18]. Endothelial cells lining veins were used as internal positive controls [18]. NCH-38 detected E-cadherin (1:50; Agilent Dako Products; Amstelveen, Noord-Holland, The Netherlands) using essentially the same protocol described for D1C2 [17]. Differences were as follows: antigen retrieval under 0.9 bar and secondary antibody E0413 (1:150; Agilent Dako Products). Squamous epithelium adjacent to the cancer was used as an internal positive control. Two observers (M.J.D.H. and B.v.d.S.) prescored all three markers. A third observer (S.M.W.) revised the scores. In case of disagreement, reevaluation was performed by M.J.D.H. and S.M.W. simultaneously until agreement was reached. Well-differentiated cancer cells that show no nuclei were omitted during scoring.

2.3.1. Scoring of D1C2, A2H2-3, and E-cadherin immunoreactivity across WTSs

Membranous immunoreactivities obtained using D1C2, A2H2-3, and NCH-38 differ markedly not only between but also within slides. Staining intensities varied from 0 to 3 [17] and were either constant across cancer fields or varied between the center and periphery of cancer fields. To characterize and organize the observed interslide and intraslide variation, staining intensities were dichotomized and assessed for both the center and periphery of cancer fields. Cancer cells showing no (0) to weak (1) basal and/or lateral membranous immunoreactivity were assessed as negative, whereas cancer cells showing moderate (2) to strong (3) basal and/or lateral membranous immunoreactivity were assessed as positive. The periphery of cancer fields was defined as *the outer 2−3 cell layers of a cancer field*. The center of cancer fields was defined as *other than the outer 2−3 cell layers of a cancer field*. To consistently assess the possible combinations of central and peripheral scores, a two-dimensional scoring system was designed that describes four staining patterns: uniform negative, gradient toward the periphery, uniform positive, and gradient toward the center (Fig. 1, Supplementary Fig. 1). Using this system, the percentage(s) of the observed staining pattern(s) was scored per cancer.

2.3.2. Scoring of MET protein status and ECD shedding across WTSs

All cancers—scored for D1C2 and A2H2-3—were assigned to one of the three categories: MET negative, MET decoy receptor, or TM MET with or without the ECD. Analogous to previous work [17,18], negative for MET was assigned when >90% of cancer cells showed absence of membranous immunoreactivity for C- and N-terminal MET. MET decoy receptor was assigned if the cancer cells showed more N-terminal than C-terminal membranous MET immunoreactivity in the form of the gradient toward the periphery and/or uniform positive staining pattern. The percentage of cancer cells showing the decoy receptor was calculated for each staining pattern by subtracting the percentage of C-terminal MET immunoreactivity from the percentage of N-terminal MET immunoreactivity. TM MET subjective or not subjective to shedding was assigned if the cancer cells showed more amounts of C-terminal or equal amounts of C-terminal than N-terminal membranous MET immunoreactivity in the form of the gradient toward the periphery and/or uniform positive staining pattern. The percentage of cancer cells subjective to shedding was calculated for each staining pattern by subtracting the percentage of N-terminal MET immunoreactivity from the percentage of C-terminal MET immunoreactivity.

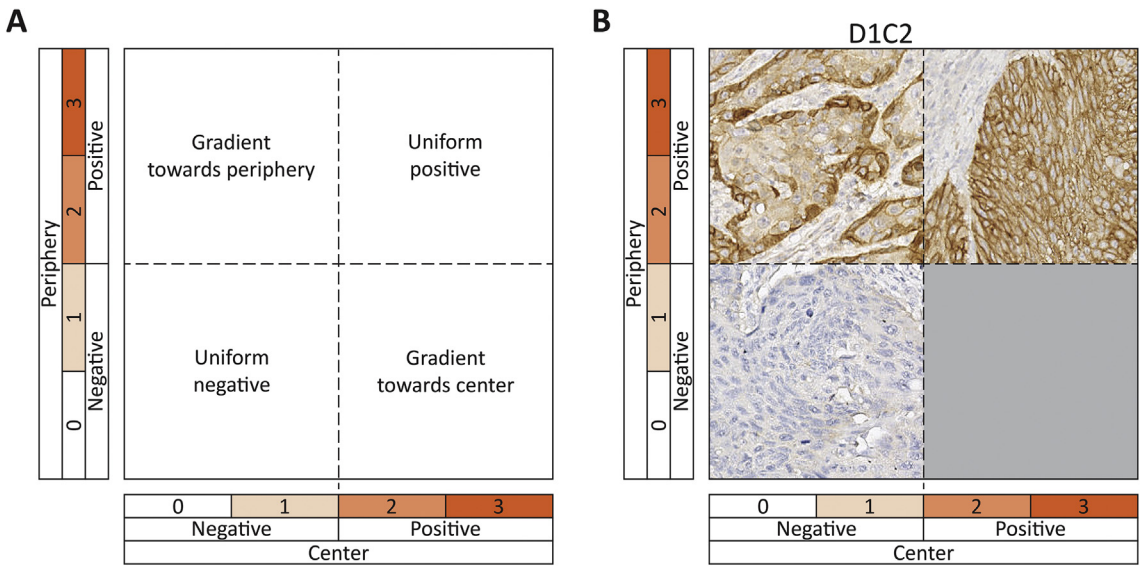

Fig. 1 Two-dimensional scoring system that characterizes four staining patterns: uniform negative, gradient toward the periphery, uniform positive, and gradient toward the center. A, Schematic representation of the two-dimensional scoring system designed to describe the four defined staining patterns. B, Photographs representing the defined staining patterns observed using D1C2 (×20 objective). For D1C2, the gradient toward the center staining pattern was not observed (indicated by a gray square).

2.4. Assessment of association between C-terminal MET, ECD shedding, loss of E-cadherin, and survival

To assess which staining pattern for D1C2, A2H2-3, and NCH-38 is most informative with respect to survival for cancers positive for TM C-terminal MET, receiver operating characteristic (ROC) curve analyses were performed for both overall survival (OS) and disease-free survival (DFS) using the area under the curve as a performance measure (Supplementary Tables 1 and 2). An identical approach was taken for ECD shedding across the gradient toward the periphery and uniform positive staining pattern (Supplementary Tables 3 and 4). The optimal cutoff values for uniform positive D1C2 immunoreactivity, uniform negative NCH-38 immunoreactivity, and ECD shedding within the D1C2 uniform positive staining pattern in view of survival analysis for both OS and DFS were determined using the maximal value of the Youden index (Supplementary tables 5 through 8 for uniform positive D1C2, Supplementary tables 9 through 12 for uniform negative NCH-38, and Supplementary tables 13 through 16 for ECD shedding within the D1C2 uniform positive staining pattern).

2.5. Survival analyses

The D1C2 uniform positive staining pattern, MET ECD shedding, and E-cadherin uniform negative staining pattern OS and DFS curves were calculated by means of the Kaplan-Meier (KM) method. The log-rank test was used to assess significance of differences in survival times. Univariable and multivariable Cox proportional hazards regression models were used to assess the prognostic value of the D1C2 uniform positive staining pattern, MET ECD shedding, the E-cadherin uniform negative staining pattern, and demographical, clinical, and histopathological characteristics. The median test and independent-samples t-test were used to confirm that inclusion of tissues fixed using nonbuffered formalin (surgically removed before 1995) had no effect on the medians and averages of the prognostically relevant staining patterns (D1C2 uniform positivity, A2H2-3 uniform negativity, and NCH-38 uniform negativity) for tissues fixed using buffered formalin. Therefore, all samples (1984−2010) were included in view of the sample size for multivariable analyses. Calculations were performed using SPSS Statistics (version 25; IBM; Armonk, NY, USA). Unless otherwise mentioned, statistical significance was set at a P-value <0.05. Definitions for OS and DFS can be found in Supplementary information.

3. Results

3.1. Performance of the novel two-dimensional scoring system

To study C- and N-terminal MET and E-cadherin immunoreactivity separately and with respect to one another in OSCC, parallel WTSs of 203 cancers were stained with D1C2, A2H2-3, and/or NCH-38 and evaluated using the developed two-dimensional scoring system. The baseline characteristics are presented in Table 1. The defined staining patterns occur in combinations within a cancer section (Supplementary Fig. 2, Supplementary Table 17). In general, the patterns of gradient toward the

Table 1 Baseline characteristics for the entire sample population (n = 203).

Clinicohistopathological characteristic	No. of patients	
	#	%
Sex		
Male	115	56.7
Female	88	43.3
Age at diagnosis (years)		
Mean (range)	63.6 (26.0 −95.0)	
Subsite		
Mucosa of the lip	1	0.50
Other and unspecified parts of the tongue	100	49.3
Alveolus and gingiva	21	10.3
Floor of the mouth	54	26.6
Palate	2	1.00
Other and unspecified parts of the mouth	25	12.3
Cancer stage[a,b]		
I	57	28.1
II	33	16.3
III	30	14.8
IV	74	36.5
Missing	9	4.40
Treatment		
Surgery	104	51.2
Surgery and radiotherapy	99	48.8

pTNM − pathological primary tumor, regional lymph node, distant metastasis; AJCC − American Joint Committee on Cancer

[a] Based on pTNM, which was assessed according to the 7th edition of the AJCC.

[b] All included patients are assessed as pM = 0 by clinical and/or histological examination.

periphery and toward the center are mutually exclusive for the MET antibodies and NCH-38 (Fig. 1B, Supplementary Fig. 1).

3.1.1. Intra-antibody comparison of staining patterns

Examination of the distributions of the observed staining patterns obtained as per antibody shows that the D1C2 uniform staining patterns—negative or positive—are more often observed across smaller patches and that the gradient toward the periphery staining pattern tends to cover bigger patches (Supplementary figures 3A through D). It also shows that A2H2-3 immunoreactivity is frequently completely absent (n = 63, 31.0%), is rarely completely positive (n = 4, 2.0%), and displays relatively smaller patches of gradient toward the periphery (Supplementary figures 3E through H). Finally, it shows that the NCH-38 uniform negative staining pattern is more often observed across smaller patches, the complete uniform positive staining pattern is often absent (n = 117, 58.0%), and—if present—the gradient toward the periphery staining pattern tends to cover larger areas of the cancer (Supplementary Fig. 3I through L).

3.1.2. Comparison of the staining pattern observed with D1C2 and NCH-38

Although pairwise comparisons of the distributions and medians of the scores of inverse staining patterns of D1C2 and NCH-38 show that there are significant differences for D1C2 uniform negative versus NCH-38 uniform positive and D1C2 gradient toward the periphery versus NCH-38 gradient toward the center staining patterns, the comparisons also show that there is no significant difference between the distributions and—identical—medians (10.0%) of the D1C2 uniform positive and NCH-38 uniform negative staining pattern (Supplementary Fig. 3A though D and I through L, Supplementary Table 18, Fig. 2A through C). These results indicate that D1C2 uniform positive patches of tumor are likely to be subjective to downregulation of E-cadherin.

3.1.3. Comparison of staining patterns observed with D1C2 and A2H2-3

Pairwise comparison of the distributions and medians of the corresponding scores per respective staining pattern for D1C2 and A2H2-3 shows that they differs significantly for the three staining patterns (Supplementary figures 3A through C and 3E through G, Supplementary Table 19). The median for uniform negativity observed using A2H2-3 (65.0%) is significantly higher than that observed using D1C2 (15.0%). The opposite is true for the uniform positive (5.0% versus 10.0%, respectively) and gradient toward the periphery staining patterns (20.0% versus 40.0%, respectively, Supplementary Fig. 3D and H, Fig. 2D through F). These results suggest that ECD shedding occurs in both the uniform positive and gradient toward the periphery fraction, resulting in overall higher uniform negativity of A2H2-3 (Supplementary Fig. 4).

3.2. Evaluation of MET protein status and its association with patient prognosis

Aligning the scores obtained for D1C2 and A2H2-3 reveals that 8.9% of the cancers (n = 18) are negative for—both C- and N-terminal—MET immunoreactivity, that 16.1% of the cancers (n = 33) show the decoy receptor in the gradient toward the periphery and/or uniform positive staining pattern, and that 74.9% of the cancers (n = 152) are positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern. Within the latter category, 19.7% of the cancers (n = 30) are not subjective to MET ECD shedding and 80.3% of the cancers (n = 122) are subjective to MET ECD shedding in the gradient toward the periphery and/or uniform positive staining pattern (Supplementary Table 20).

KM curves reveal that there is no difference in OS or DFS for patients diagnosed with cancers showing absence of MET immunoreactivity, the MET decoy receptor, or TM C-terminal MET (Supplementary Fig. 5A and B).

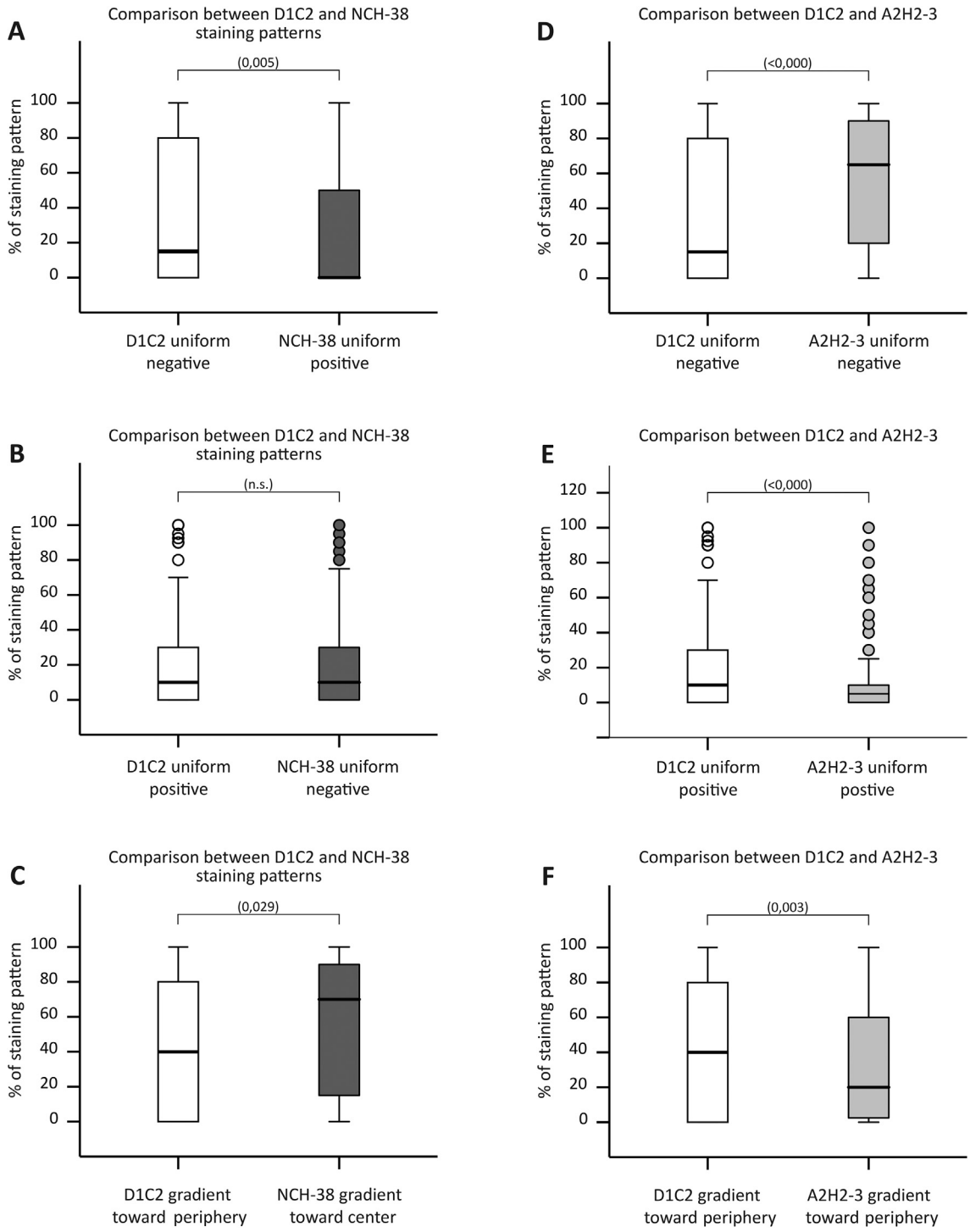

Fig. 2 Box plots illustrating statistically significant differences/similarities between the medians of the scores of inverse staining patterns observed for D1C2 and NCH-38 indicative of EMT and statistically significant differences between the medians of the scores of corresponding staining patterns observed for D1C2 and A2H2-3 indicative of MET ECD shedding as per the median test (n = 203). Statistical significance was set at $P < 0.05$. A, The median for uniform negativity observed using D1C2 (15.0%) is significantly higher than that observed for uniform positivity of NCH-38 (0.00%), implying that C-terminal MET immunoreactivity is generally lower than E-cadherin immunoreactivity. B, The medians for uniform positivity observed using D1C2 and uniform negativity observed using NCH-38 are identical (10.0%), implying that E-cadherin tends to be absent in uniform positive C-terminal MET cancer areas. C, The median for gradient toward the periphery using D1C2 (40.0%) is significantly lower than that observed for gradient toward the center using NCH-38 (70.0%), corresponding with the observation that C-terminal MET immunoreactivity is generally lower than E-cadherin immunoreactivity described under panel A. D, The median for uniform negativity observed using A2H2-3 (65.0%) is significantly higher than that observed using D1C2 (15.0%). E, The median for uniform positivity observed using A2H2-3 (5.00%) is significantly lower than that observed using D1C2 (10.0%). F, The median for gradient toward the periphery observed using A2H2-3 (20.0%) is significantly lower than that observed using D1C2 (40.0%). ECD, ectodomain; EMT, epithelial-to-mesenchymal transition.

3.3. Association of the staining patterns with patient prognosis in TM C-terminal MET−positive cancers

ROC curve analyses show that the D1C2 uniform positive and the NCH-38 uniform negative staining patterns are associated with both OS and DFS if they comprise ≥10% of cancer cells (Supplementary Figs. 6 and 7 for D1C2 and Supplementary Figs. 8 and 9 for NCH-38).

3.3.1. Prognostic value of D1C2 uniform positivity

Univariable survival analyses performed for D1C2 uniform positivity and the histopathological characteristics listed in Supplementary Table 21 show that patients showing uniform positivity for D1C2 (n = 105, 69.1%) perform significantly worse in terms of OS (hazard ratio [HR] = 2.40; 95% confidence interval [CI] = 1.25 to 4.61; and $P = 0.008$; Fig. 3A) and DFS (HR = 1.83; 95% CI = 1.07−3.14; $P = 0.027$; Fig. 3B). To test the independent value of D1C2 uniform positivity for OS and DFS, multivariable analyses were performed correcting for age at diagnosis, pT, pN, extranodal extension, and degree of differentiation. The results show that uniform positivity of D1C2 remains significantly associated with survival (HR = 2.61; 95% CI = 1.20 to 5.69; and $P = 0.016$ for OS and HR = 1.99; 95% CI = 1.05 to 3.74; and $P = 0.034$ for DFS; Table 2).

3.3.2. Prognostic value of NCH-38 uniform negativity

Univariable survival analyses show that patients showing loss of NCH-38 (n = 84, 55.3%) perform significantly worse in terms of OS (HR = 2.21; 95% CI = 1.30 to 3.77; and $P = 0.004$; Fig. 3C) and DFS (HR = 1.90; 95% CI = 1.20−3.01; $P = 0.007$; Fig. 3D). To test the independent value of NCH-38 uniform negativity for OS and DFS, multivariable analyses were performed correcting for age at diagnosis, pT, pN, extranodal extension, and degree of differentiation. The results show that uniform negativity of NCH-38 remains significantly associated with survival (HR = 2.53; 95% CI = 1.35 to 4.73; and $P = 0.004$ for OS and HR = 2.13; 95% CI = 1.26 to 3.60; and $P = 0.005$ for DFS; Table 3).

3.4. Association of MET ECD shedding with patient prognosis in TM C-terminal MET−positive cancers

ROC curve analyses show that MET ECD shedding within the D1C2 uniform positive staining pattern is associated with both OS and DFS, if it comprises ≥10% of cancer cells (Supplementary Figs. 10 and 11).

3.4.1. Prognostic value of MET ECD shedding within the D1C2 uniform positive staining pattern

Univariable survival analyses show that patients showing ECD shedding within the D1C2 uniform positive staining pattern (n = 66, 43.4%) perform significantly worse in terms of OS (HR = 2.30; 95% CI = 1.38 to 3.83; and $P = 0.001$; Fig. 3E) and DFS (HR = 1.87; 95% CI = 1.19−2.92; $P = 0.006$; Fig. 3F). To test the independent value of ECD shedding within the D1C2 uniform positive staining pattern for OS and DFS, multivariable analyses were performed correcting for age at diagnosis, pT, pN, extranodal extension, and degree of differentiation. The results show that MET ECD shedding remains significantly associated with survival (HR = 2.39; 95% CI = 1.29 to 4.43; and $P = 0.006$ for OS and HR = 1.94; 95% CI = 1.14 to 3.30; and $P = 0.015$ for DFS; Table 4).

4. Discussion

Although MET is an interesting target for therapy [5−7], its status as a biomarker is unclear, and there is a lack of appropriate CDx [9,12,13,15]. Using TMAs, it was shown that C-terminal MET immunoreactivity and shedding are prognostically informative for OSCC [17,18]. The present study shows that these results can be extrapolated to WTSs using a novel two-dimensional scoring system.

The scoring system divides the variable staining pattern into two categories—gradient toward the periphery and gradient toward the center—which are mutually exclusive for both MET antibodies (D1C2 and A2H2-3) and the E-cadherin antibody (NCH-38). This is expected as transcription of MET is induced by hepatocyte growth factor (HGF) [21], which is produced by fibroblasts residing in the stromal compartment of cancers [22,23]. MET itself facilitates transcriptional downregulation of E-cadherin through transcription factors such as Snail/SNAI1 [24]. Such transcriptional downregulation of E-cadherin also provides an explanation for the associations observed between D1C2 uniform positivity and NCH-38 uniform negativity. Besides EMT, the developed scoring system also allows investigation of MET protein status (no, decoy, TM C-terminal positive) and ECD shedding in TM C-terminal MET−positive cancers by aligning D1C2 and A2H2-3 staining patterns.

Assuming that TM C-terminal MET−positive cancers are eligible for treatment with biologicals directed against MET, it was examined within this specific group whether the defined MET staining patterns and ECD shedding show a relation with survival using ROC curve analyses. As these staining patterns are highly related to one another, it was decided beforehand that only the most informative pattern per marker would be used in a final—multivariable—survival model to avoid colinearity. This approach resulted in the thresholds of ≥10% for D1C2 uniform positivity and ≥10% of ECD shedding within uniform positive patches of D1C2. The absence of an association between N-terminal MET immunoreactivity and survival is consistent with prior results [18]. Using the same methodology, a relation was established between ≥10% of NCH-38 uniform negativity and survival. Considering the

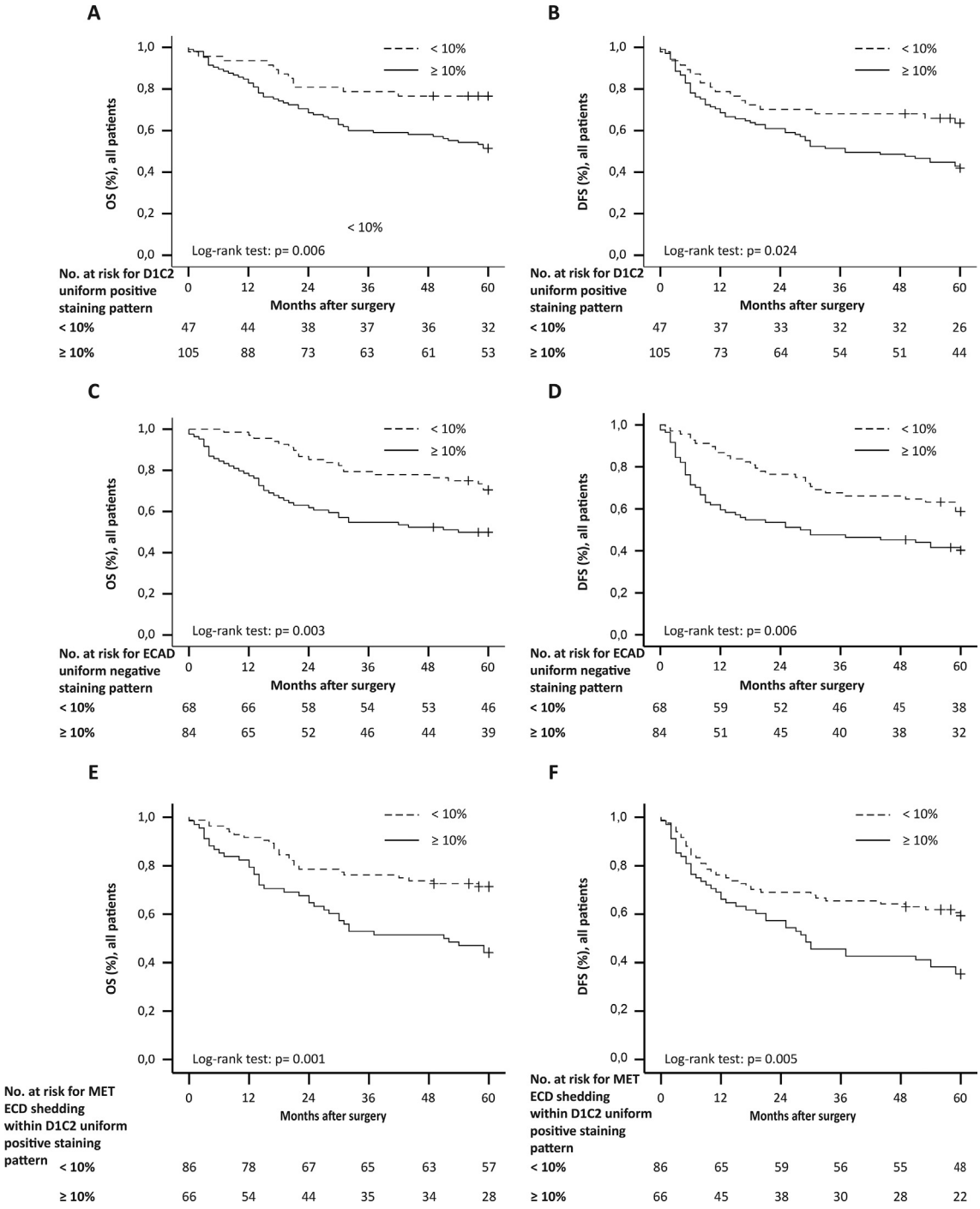

Fig. 3 **KM curves**. A, OS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by D1C2 uniform positivity. B, DFS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by D1C2 uniform positivity. C, OS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by NCH-38 uniform negativity. D, DFS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by NCH-38 uniform negativity. E, OS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by MET ECD shedding within the D1C2 uniform positive staining pattern. F, DFS for patients positive for TM C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152), stratified by MET ECD shedding within the D1C2 uniform positive staining pattern. ECD, ectodomain; TM, transmembranous; DFS, disease-free survival; OS, overall survival; KM, Kaplan-Meier.

Table 2 Multivariable analysis—in view of the D1C2 uniform positive staining pattern—of overall survival and disease-free survival for patients having cancers that are positive for transmembranous C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152).

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age at diagnosis[a]	1.39	1.10–1.74	**0.005**	1.27	1.05–1.55	**0.015**
pT ≥ 1	1.53	0.79–2.97	0.206	1.18	0.68–2.03	0.560
pN ≥ 2	2.65	1.34–5.23	**0.005**	1.95	1.03–3.68	**0.040**
Extranodal extension present	1.19	0.54–2.62	0.671	1.00	0.47–2.14	0.994
Poor − undifferentiated opposed to well − moderate	0.95	0.45–2.00	0.887	0.80	0.40–1.61	0.530
≥10% of cancer cells show the D1C2 uniform positive staining pattern	2.61	1.20–5.69	**0.016**	1.99	1.05–3.74	**0.034**

Abbreviations: HR, hazard ratio; CI, confidence interval.

The bold values are smaller than 0.05 indicating statistical significance.
[a] The HR was based on 10-year intervals.

Table 3 Multivariable analysis—in view of the NCH-38 uniform negative staining pattern—of overall survival and disease-free survival for patients having cancers that are positive for transmembranous C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152).

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age at diagnosis[a]	1.42	1.14–1.77	**0.002**	1.36	1.11–1.65	**0.003**
pT ≥ 1	1.43	0.73–2.82	0.300	1.15	0.66–2.01	0.615
pN ≥ 2	2.76	1.36–5.59	**0.005**	2.10	1.10–4.01	**0.024**
Extranodal extension present	0.91	0.40–2.05	0.814	0.89	0.41–1.92	0.768
Poor − undifferentiated opposed to well − moderate	1.19	0.56–2.50	0.652	0.91	0.45–1.82	0.785
≥10% of cancer cells show the NCH-38 uniform negative staining pattern	2.53	1.35–4.73	**0.004**	2.13	1.26–3.60	**0.005**

Abbreviations: HR, hazard ratio; CI, confidence interval.

The bold values are smaller than 0.05 indicating statistical significance.
[a] The HR was based on 10-year intervals.

Table 4 Multivariable analysis—in view of ectodomain shedding within the D1C2 uniform positive staining pattern—of overall survival and disease-free survival for patients having cancers positive for transmembranous C-terminal MET in the gradient toward the periphery and/or uniform positive staining pattern (n = 152).

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age at diagnosis[a]	1.32	1.05–1.66	**0.017**	1.24	1.02–1.51	**0.032**
pT ≥ 1	1.78	0.91–3.49	0.092	1.32	0.76–2.29	0.325
pN ≥ 2	2.39	1.17–4.91	**0.017**	1.84	0.95–3.57	0.071
Extranodal extension present	1.20	0.53–2.72	0.661	1.02	0.47–2.20	0.967
Poor − undifferentiated opposed to well − moderate	0.95	0.44–2.05	0.894	0.79	0.39–1.61	0.513
≥10% of cancer cells undergo ectodomain shedding in the D1C2 uniform positive staining pattern	2.39	1.29–4.43	**0.006**	1.94	1.14–3.30	**0.015**

Abbreviations: HR, hazard ratio; CI, confidence interval.

The bold values are smaller than 0.05 indicating statistical significance.
[a] The HR was based on 10-year intervals.

sample size, uniform positivity of D1C2, ECD shedding, and uniform negativity of NCH-38 were corrected using the same six variables used to correct for ECD shedding in the TMA study [18], more specifically age at diagnosis, pT, pN, extranodal growth, and degree of differentiation, all of which are known to be associated with survival [25–28]. Because of the earlier observed interaction between vasoinvasive growth and the D1C2 uniform staining patterns [17], interaction between vasoinvasive growth and D1C2 uniform positivity or ECD shedding was excluded (results not shown). In addition, overcorrection by including both degree of differentiation and uniform negativity of E-cadherin in a single multivariable model was also excluded (results not shown).

There are some discrepancies between the results observed using the TMA and WTSs. Using WTSs, there is no association between absence of C-terminal MET immunoreactivity and survival. In contrast to the TMA study [17], wherein each cancer was scored for one staining pattern, the WTSs can show combinations of staining patterns. This implies that although such combinations are not observed while scoring the TMA, they could be represented in the sampling tissue. Therefore, the earlier observed association of absence of C-terminal MET with poor survival is probably due to another—uniform positive—staining pattern not sampled during TMA production. Moreover, there is a difference in the thresholds set for D1C2 uniform positivity. This might be so because the TMA threshold was set including all cancers evaluated for D1C2 immunoreactivity (negative and positive). Although including only C-terminal MET−positive OSCC lowers the TMA threshold for uniform positivity, it remains higher than the threshold set for WTSs (results not shown). This is also the case for ECD shedding. Similar to the lack of association between uniform negativity and survival for WTSs, we argue that these differences in thresholds—for both D1C2 uniform positivity and ECD shedding—are likely due to TMA sampling and tumor heterogeneity [17,18]. Finally—in contrast to the TMA results—the WTS study shows that shedding is not only associated with DFS but also associated with OS. Because shedding is determined within D1C2 uniform positive tumor patches for the WTS study, this finding is in line with the TMA result, showing that uniform positivity is associated with OS and DFS [17,18].

Despite these differences, the study shows that other findings are consistent with prior results. The uncorrected HRs for both D1C2 uniform positivity and ECD shedding are of the same order of magnitude for the WTSs and TMA studies (Supplementary Tables 22 and 23). In addition, the HR found for OS for the uniform negative NCH-38 staining pattern is comparable with the HR reported for loss of E-cadherin described in a meta-analysis concerning E-cadherin immunoreactivity in OSCC [29]. It is therefore concluded that the developed scoring system provides valid results. Established use of scoring systems [30] and/or CDx [31,32] using patterns and intensity scoring in the field of pathology indicates that it is feasible to implement the developed scoring system in a—oral cancer—diagnostic setting.

The observation that C-terminal MET uniform positivity and ECD shedding are independently associated with poor OS and DFS—independent of the disease stage (Supplementary Tables 24 and 25) in TM C-terminal MET−positive OSCC—concurs with the fact that ECD shedding has been described to increase the malignant potential of the MET oncogene [33]. Moreover, it suggests that MET is a promising target for therapy. However, low success rates of performed clinical trials led to the belief that immunohistochemistry is inadequate for patient stratification. Instead, it is argued that stratification should be

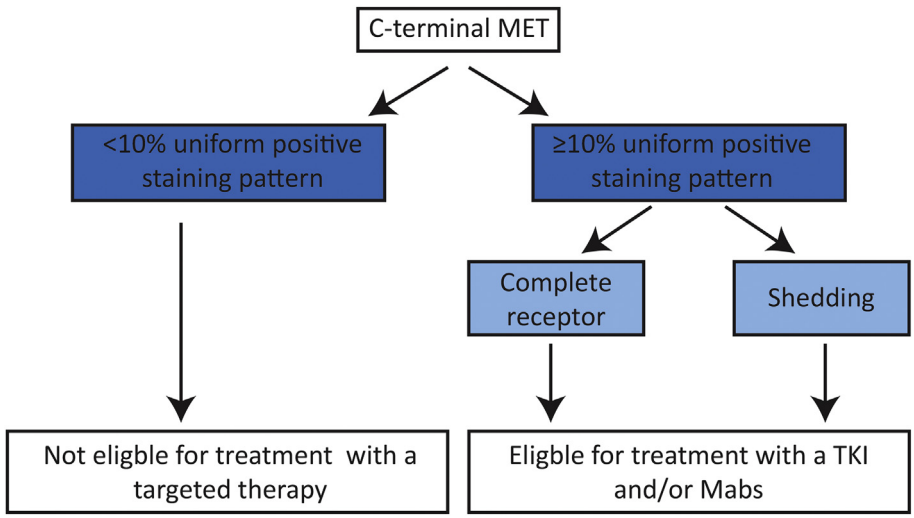

Fig. 4 Proposed stratification scheme for OSCC eligible or not eligible for targeted therapies directed against MET. OSCC, oral squamous cell carcinoma; TKI: tyrosine kinase inhibitor; Mabs: monoclonal antibody.

based on *MET* genetic aberrations, such as amplification, point mutations, exon 14 skipping, and oncogenic fusions [34,35]. Indeed, recent trials implementing patient selection based on genetic alterations show initial successes in tumor reduction. However, inhibition of wild-type MET activity has been shown to reduce cell survival, local invasion, and distant metastasis. This makes wild-type MET a suitable target for adjuvant therapy after curative primary surgery as its targeting potentially eradicates residual cancer cells [34]. Taking everything into consideration and knowing that reliable antibodies were used, we think that the results presented here could be of added value in the development of CDx (Fig. 4).

The developed scoring system characterizes D1C2, A2H2-3, and NCH-38 immunoreactivity across cancer sections in the form of staining patterns (uniform negative or positive and gradient toward the periphery or center). By aligning D1C2, A2H2-3, and/or NCH-38 staining patterns, it also facilitates investigation of MET protein status and biological processes such as MET ECD shedding and EMT. Finally, it establishes an independent association of D1C2 uniform positivity, ECD shedding, and loss of E-cadherin with poor OS and DFS. Ultimately, the findings concerning MET immunoreactivity and ECD shedding might support the development of CDx for targeted therapies directed against the RTK MET or orchestrators of shedding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2020.07.018.

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References

[1] NCCN. NCCN Clinical practice guidelines in oncology, https://www.nccn.org/professionals/physician_gls/pdf/aml.pdf [accessed 6 May 2019].

[2] Amit M, Yen TC, Liao CT, et al. International Consortium for Outcome Research in H, Neck C. Improvement in survival of patients with oral cavity squamous cell carcinoma: an international collaborative study. Cancer 2013;119:4242–8. https://www.ncbi.nlm.nih.gov/pubmed/24114787.

[3] Genden EM, Ferlito A, Silver CE, et al. Contemporary management of cancer of the oral cavity. Eur Arch Oto-Rhino-Laryngol 2010;267:1001–17. https://www.ncbi.nlm.nih.gov/pubmed/20155361.

[4] Oliver RJ, Clarkson JE, Conway DI, et al. Interventions for the treatment of oral and oropharyngeal cancers: surgical treatment. Cochrane Database Syst Rev 2007:CD006205. https://www.ncbi.nlm.nih.gov/pubmed/17943894.

[5] Lorch JH, Posner MR, Wirth LJ, Haddad RI. Seeking alternative biological therapies: the future of targeted molecular treatment. Oral Oncol 2009;45:447–53. https://www.ncbi.nlm.nih.gov/pubmed/19027348.

[6] Gentile A, Trusolino L, Comoglio PM. The Met tyrosine kinase receptor in development and cancer. Canc Metastasis Rev 2008;27:85–94. https://www.ncbi.nlm.nih.gov/pubmed/18175071.

[7] Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Canc 2018;18:269–82. https://www.ncbi.nlm.nih.gov/pubmed/29497144.

[8] De Herdt MJ, Baatenburg de Jong RJ. HGF and c-MET as potential orchestrators of invasive growth in head and neck squamous cell carcinoma. Front Biosci 2008;13:2516–26. https://www.ncbi.nlm.nih.gov/pubmed/17981731.

[9] Szturz P, Budikova M, Vermorken JB, et al. Prognostic value of c-MET in head and neck cancer: a systematic review and meta-analysis of aggregate data. Oral Oncol 2017;74:68–76. https://www.ncbi.nlm.nih.gov/pubmed/29103754.

[10] Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Canc 2004;4:118–32. https://www.ncbi.nlm.nih.gov/pubmed/14964308.

[11] Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 2003;4:657–65. https://www.ncbi.nlm.nih.gov/pubmed/12923528.

[12] Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Canc 2012;12:89–103. https://www.ncbi.nlm.nih.gov/pubmed/22270953.

[13] Huang F, Ma Z, Pollan S, et al. Quantitative imaging for development of companion diagnostics to drugs targeting HGF/MET. J Pathol Clin Res 2016;2:210–22. https://www.ncbi.nlm.nih.gov/pubmed/27785366.

[14] Kim KH, Kim H. Progress of antibody-based inhibitors of the HGF-cMET axis in cancer therapy. Exp Mol Med 2017;49:e307. https://www.ncbi.nlm.nih.gov/pubmed/28336955.

[15] Administration Usfd. Companion Diagnostics, https://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm407297.htm [accessed 26 February 2019].

[16] Lefebvre J, Ancot F, Leroy C, Muharram G, Lemiere A, Tulasne D. Met degradation: more than one stone to shoot a receptor down. Faseb J 2012;26:1387–99. http://www.ncbi.nlm.nih.gov/pubmed/22223753.

[17] De Herdt MJ, Willems SM, van der Steen B, et al. Absent and abundant MET immunoreactivity is associated with poor prognosis of patients with oral and oropharyngeal squamous cell carcinoma.

Oncotarget 2016;7:13167−81. https://www.ncbi.nlm.nih.gov/pubmed/26909606.
[18] De Herdt MJ, Koljenovic S, van der Steen B, et al. MET ectodomain shedding is associated with poor disease-free survival of patients diagnosed with oral squamous cell carcinoma. Mod Pathol 2020;33:1015−32. https://doi.org/10.1038/s41379-019-0426-2.
[19] Federa. Codes of Conduct, https://www.federa.org/codes-conduct [accessed 10 January 2019].
[20] American Joint Commity on Cancer. AJCC cancer staging manual. 7th ed. New York: Springer; 2010.
[21] Boccaccio C, Gaudino G, Gambarotta G, Galimi F, Comoglio PM. Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. J Biol Chem 1994;269:12846−51. https://www.ncbi.nlm.nih.gov/pubmed/8175699.
[22] Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 1987;327:239−42. https://www.ncbi.nlm.nih.gov/pubmed/2952888.
[23] Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Canc 2002;2:289−300. https://www.ncbi.nlm.nih.gov/pubmed/12001990.
[24] Cano A, Perez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000;2:76−83. https://www.ncbi.nlm.nih.gov/pubmed/10655586.
[25] Ding D, Stokes W, Eguchi M, et al. Association between lymph node ratio and recurrence and survival outcomes in patients with oral cavity cancer. JAMA Otolaryngol Head Neck Surg 2019;145:53−61. https://www.ncbi.nlm.nih.gov/pubmed/30452499.
[26] Mermod M, Tolstonog G, Simon C, Monnier Y. Extracapsular spread in head and neck squamous cell carcinoma: a systematic review and meta-analysis. Oral Oncol 2016;62:60−71. https://www.ncbi.nlm.nih.gov/pubmed/27865373.
[27] Saggi S, Badran KW, Han AY, Kuan EC, St John MA. Clinicopathologic characteristics and survival outcomes in floor of mouth squamous cell carcinoma: a population-based study. Otolaryngol Head Neck Surg 2018;159:51−8. https://www.ncbi.nlm.nih.gov/pubmed/29436280.
[28] Xu Q, Wang C, Li B, et al. The impact of age on oral squamous cell carcinoma: A longitudinal cohort study of 2,782 patients. Oral Dis 2019;25:730−41. https://doi.org/10.1111/odi.13015.
[29] Zhao Z, Ge J, Sun Y, et al. Is E-cadherin immunoexpression a prognostic factor for head and neck squamous cell carcinoma (HNSCC)? A systematic review and meta-analysis. Oral Oncol 2012;48:761−7. https://www.ncbi.nlm.nih.gov/pubmed/22455948.
[30] Epstein JI, Egevad L, Amin MB, et al. The 2014 international society of urological pathology (ISUP) consensus conference on gleason grading of prostatic carcinoma: definition of grading patterns and proposal for a new grading system. Am J Surg Pathol 2016;40:244−52. https://www.ncbi.nlm.nih.gov/pubmed/26492179.
[31] Dako. HercepTest™ Interpretation Manual Breat Cancer, https://www.agilent.com/cs/library/usermanuals/public/28630_herceptest_interpretation_manual-breast_ihc_row.pdf [accessed 26 February 2020].
[32] Roche. Guiding immunotherapy decisions, https://diagnostics.roche.com/global/en/products/tests/ventana-pd-l1-_sp142-assay1.html [accessed 26 February 2020].
[33] Merlin S, Pietronave S, Locarno D, Valente G, Follenzi A, Prat M. Deletion of the ectodomain unleashes the transforming, invasive, and tumorigenic potential of the MET oncogene. Canc Sci 2009;100:633−8. https://www.ncbi.nlm.nih.gov/pubmed/19175607.
[34] Comoglio PM, Trusolino L, Boccaccio C. Known and novel roles of the MET oncogene in cancer: a coherent approach to targeted therapy. Nat Rev Canc 2018;18:341−58. https://www.ncbi.nlm.nih.gov/pubmed/29674709.
[35] Koch JP, Aebersold DM, Zimmer Y, Medova M. MET targeting: time for a rematch. Oncogene 2020;39:2845−62. https://www.ncbi.nlm.nih.gov/pubmed/32034310.