The Photon Islam and Neck Cancer Testings Predict Response to Immune Checkpoint-Blockade in Head and

Neck Cancer

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Abstract:

IMMETHET ALTERT INTERT INTERT INTERT AUTHET AUTHET AUTHET AND THE RET AND THE RET AND THE RET IS THE RET IS THE RET IS THE RET IS IN THE RET IS IN THE RET IS IN THE STANDARD IN THE STANDARD OF THE STANDARD OF THE STANDARD necting the likelihood of response to ICB is a single proportional biomarker (PD-L1) expressed
in immune and tumor cells (Combined Positive Score, CPS) without differentiation by cell type,
potentially explaining its limit predicting the limit of response to a single proportional tiferentiation by cell type,
in immune and tumor cells (Combined Positive Score, CPS) without differentiation by cell type,
potentially explaining its limited predi potentially explaining its limited predictive value. Tertiary Lymphoid Structures (TLS) have
shown a stronger association with ICB response than PD-L1. However, their exact composition,
size, and spatial biology in HNSCC r prediating explaining in antital predictive transformation, explained the sixter (FLS) and
shown a stronger association with ICB response than PD-L1. However, their exact composition,
size, and spatial biology in HNSCC rem size, and spatial biology in HNSCC remain understudied. A detailed understanding of TLS is
required for future use as a clinically applicable predictive biomarker. Methods: Pre-ICB tumor
tissue sections were obtained from start paint and spatial biomated predictive biomarker. Methods: Pre-ICB tumor
tissue sections were obtained from 9 responders (complete response, partial response, or
stable disease) and 11 non-responders (progressive dise required for future use as a clinically applicable predictive biomarker. Methods: The leb tumor
tissue sections were obtained from 9 responders (complete response, partial response, or
stable disease) and 11 non-responders Istable disease) and 11 non-responders (progressive disease) classified via RECISTV1.1. A custom
multi-immunofluorescence (mIF) staining assay was designed, optimized, and applied to
characterize tumor cells (pan-cytokera multi-immunofluorescence (mIF) staining assay was designed, optimized, and applied to
characterize tumor cells (pan-cytokeratin), T cells (CD4, CD8), B cells (CD19, CD20), myeloid cells
(CD16, CD56, CD163), dendritic cell characterize tumor cells (pan-cytokeratin), T cells (CD4, CD8), B cells (CD19, CD20), myeloid cells
(CD16, CD56, CD163), dendritic cells (LAMP3), fibroblasts (α Smooth Muscle Actin), proliferative
status (Ki67) and immuno CD16, CD56, CD163), dendritic cells (LAMP3), fibroblasts (α Smooth Muscle Actin), proliferative
status (Ki67) and immunoregulatory molecules (PD1). Spatial metrics were compared among
groups. Serial tissue sections wer Status (Ki67) and immunoregulatory molecules (PD1). Spatial metrics were compared among
groups. Serial tissue sections were scored for TLS in both H&E and mIF slides. A machine
learning model was employed to measure the e status (REP) and immunoregulatory motional (POL) epartmentic and miff-slides. A machine
groups. Serial tissue sections were scored for TLS in both H&E and miff-slides. A machine
learning model was employed to measure the earning model was employed to measure the effect of these metrics on achieving a response
to ICB (SD, PR, or CR). **Results:** A higher density of B lymphocytes (CD20+) was found in
responders compared to non-responders to to ICB (SD, PR, or CR). Results: A higher density of B lymphocytes (CD20+) was found in
responders compared to non-responders to ICB (p=0.022). A positive correlation was observed
between mIF and pathologist identificatio to ICB (SD, FR, OF CR). Results: A higher density of B lymphocytes (CD20+) was found in
responders compared to non-responders to ICB (p=0.022). A positive correlation was observed
between mIF and pathologist identificatio between mIF and pathologist identification of TLS ($R^2 = 0.66$, p -value= <0.0001). TLS trended
toward being more prevalent in responders to ICB (p=0.0906). The presence of TLS within 100
um of the tumor was associated w between mIF and pathologist identification of TLS (R
toward being more prevalent in responders to ICB (p=
µm of the tumor was associated with improved overa
(p=0.03). A multivariate machine learning model identi
response t $= 0.66$, p value= $\infty.0001$, $\infty.0001$ and progression-free survival fied TLS density as a leading predictor of the cell densities and TLS spatial location in the immune response to HNSCC and sponse. the terms are presented with improved overall (p=0.04) and progression-free survival

(p=0.03). A multivariate machine learning model identified TLS density as a leading predictor of

response to ICB with 80% accuracy. Con potentially outperform change in the tumor within the tumor microenvironment play a critical role in the immune cell densities and TLS spatial location
within the tumor microenvironment play a critical role in the immune r (p=0.03). A multimate mathematic mathematic mathematic reasons to ICB with 80% accuracy. Conclusion: Immune cell densities and TLS spatial location within the tumor microenvironment play a critical role in the immune respo response to ICB with 80% accuracy. Conclusion: Immune cell densities and TLS spatial location
within the tumor microenvironment play a critical role in the immune response to HNSCC and
may potentially outperform CPS as a p within the tumor microenvironment play a critical role in the immune response.

may potentially outperform CPS as a predictor of ICB response. may potentially outperform CPS as a predictor of ICB response.

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Introduction:

Morldwide and accounts for 4.5% of cancer diagnoses and deaths (1,2). In the United States, 15% of cases present with distant metastasis, for which the 5-year survival rate is 39.3% (3). Immune checkpoint blockade (ICB), w 15% of cases present with distant metastasis, for which the 5-year survival rate is 39.3% (3).

Immune checkpoint blockade (ICB), with or without chemotherapy, is the standard of care for

recurrent or metastatic HNSCC, bu 15% of cases present with distant metastasis), or minimized year survivaled and of care for
15% of extent metastatic HNSCC, but only 20% of patients achieve a clinical benefit (4–6). The
15% of patients achieve a clinical Immune, and stromal cells- among all cells in a single section of tumor tissue. The Combined Positive Score (CPS) is the proportion of all PD-L1 positive cells-including tumor, immune, and stromal cells- among all cells in Combined Positive Score (CPS) is the proportion of all PD-L1 positive cells-including tumor,
immune, and stromal cells- among all cells in a single section of tumor tissue. The CPS is
calculated manually by a pathologist a Entrance Positive Combined Positive Comparison of the proportion of tumor tissue. The CPS is
calculated manually by a pathologist and is often used to assist with selecting a treatment
modality and estimating prognosis (7) implementated manually by a pathologist and is often used to assist with selecting a treatment
modality and estimating prognosis (7). However, CPS has limited performance in predicting
response to treatment (8). Therefore, modality and estimating prognosis (7). However, CPS has limited performance in predicting
response to treatment (8). Therefore, an unmet need exists for accurate predictive biomarkers
of response to ICB in HNSCC.
Tertiary

modeling progression (8). Therefore, an unmet need exists for accurate predictive biomarkers
of response to ICB in HNSCC.
Tertiary Lymphoid Structures (TLS) are lymphoid aggregates in non-lymphoid tissues and are
commonly retiary Lymphoid Structures (TLS) are lymphoid aggregates in non-lymphoid tissues and are
commonly associated with chronic inflammation and cancer (9). Their presence in the tumor
microenvironment (TME) provides evidence o Tertiary Lymphoid Structures
commonly associated with c
microenvironment (TME) pro
and adaptive arms of the im
and higher responses to ICB
proven to predict response Commonly associated with chronic inflammation and cancer (9). Their presence in the tumor
microenvironment (TME) provides evidence of intratumoral cooperation between the innate
and adaptive arms of the immune and are ofte microenvironment (TME) provides evidence of intratumoral cooperation between the innate
and adaptive arms of the immune and are often associated with improved clinical outcomes
and higher responses to ICB in solid malignan and adaptive arms of the immune and are often associated with improved clinical outcomes
and higher responses to ICB in solid malignancies (10–13). Recently, a TLS gene signature was
proven to predict response to ICB in HN and higher responses to ICB in solid malignancies (10–13). Recently, a TLS gene signature was
proven to predict response to ICB in HNSCC (14). However, their composition, size, spatial
organization, and distribution within and hydrogen to predict response to ICB in HNSCC (14). However, their composition, size, spatial organization, and distribution within and across tumor sites in HNSCC patients treated with ICB remain understudied. proven to prediction within and across tumor sites in HNSCC patients treated with ICB remain understudied.

Framain understudied. organization, and distribution, and across tumor sites in the sites in HNSCC patients treated with ICBS patients treated with ICBS patients treated with ICBS patients treated with ICBS patients transition with ICBS patient

malignancies (15,16). It allows for the spatial characterization of protein markers without
altering the tissue architecture, enabling deep analysis of cell-cell spatial interaction in the TME
(17). Newer scanning and imag altering the tissue architecture, enabling deep analysis of cell-cell spatial interaction in the TME

(17). Newer scanning and image analysis platforms facilitate the use of this technology on a

larger scale (18). Here, w altering the tissue architecture, enably one architecture, enables the tissue of this technology on a larger scale (18). Here, we applied high-plex imaging technology to profile the TME of pre-
treatment tumor specimens of

Materials and Methods:

(17). Harger scale (18). Here, we applied high-plex imaging technology to profile the TME of pre-
treatment tumor specimens of HNSCC receiving ICB.
Materials and Methods:
Patient selection: After Institutional Review Board (FFPE) tissue blocks before ICB start were collected and processed for multiplex Materials and Methods:

Patient selection: After Institutional Review Board approximate School CB.

The Hospital School of Hospital School CB.

(FFPE) tissue blocks before ICB start were approximately retrospectively selected based on response status. Nine responders and eleven non-responders
to ICB, classified via RECISTv1.1 criteria, were included. Formalin-fixed paraffin-embedded
(FFPE) tissue blocks before ICB start retrospectively selected and response in a response on response to ICB, classified via RECISTv1.1 criteria, were included. Formalin-fixed paraffin-embedded (FFPE) tissue blocks before ICB start were collected and processed (FFPE) tissue blocks before ICB start were collected and processed for multiplex
immunofluorescence (mIF) staining. Patients CTC-18, 1405_ICI-9, 1431-CTC-21, and 1408_ICI-
25 had 1 replicate from the same specimen. Replica (FFF) start and processed (mIF) staining. Patients CTC-18, 1405_ICI-9, 1431-CTC-21, and 1408_ICI-25 had 1 replicate from the same specimen. Replicates were included in the spatial analysis (n=24). Spatial data from the sli

conventional immunohistochemistry (IHC), multiplex validation, and assay optimization. Before 24). Spatial data from the slides with the biggest tissue size were included in the survival
analysis and the prediction model (n=20).
FFPE tissue specimens: Serially cut 4-µm thick sections were obtained from FFPE tumo (n=20).

FFPE tissue specimens: Serially cut 4-µm thick sections were obtained from FFPE tumor

biopsies. Human tonsils and discarded HNSCC FFPE tissue blocks were prepared for

conventional immunohistochemistry (IHC), mul FFPE tissue specimens: Serially cut $4-\mu$
biopsies. Human tonsils and discarded
conventional immunohistochemistry (IHC)
staining, all tissue slides were deparaffini:
ethanol concentrations. FFFE tissue specimens: Serially cut 4-µm thick sections were obtained from FFFE tumor
biopsies. Human tonsils and discarded HNSCC FFPE tissue blocks were prepared for
conventional immunohistochemistry (IHC), multiplex vali biomstrained immunohistochemistry (IHC), multiplex validation, and assay optimization. Before staining, all tissue slides were deparaffinized and rehydrated by serial passage through graded ethanol concentrations. conventional immunohistochemistry (IHC), multiplex validation, and assay operations setting, all tissue slides were deparaffinized and rehydrated by serial passage through graded ethanol concentrations. ethanol concentrations.

Immunomistochemistry (IHC) validation. Single tumor sections were stained with emologen

based IHC to validate our multiplexed immunofluorescence staining. All staining was manually

performed, with antibodies against the performed, with antibodies against the following: Pancytokeratin AE1/AE3
(Leica/(AE3/AE3)/Mouse-IgG1, 225 mg/L), CD19 (Leica/BT51E/Mouse-IgG2B, 35 mg/L), CD56
(Leica/CD564/Mouse, 11 mg/L), CD16 (Cell Signaling/D1N9L/Rabbit (Leica/(AE3/AE3)/Mouse-IgG1, 225 mg/L), CD19 (Leica/BT51E/Mouse-IgG2B, 35 mg/L), CD56
(Leica/CD564/Mouse, 11 mg/L), CD16 (Cell Signaling/D1N9L/Rabbit IgG, 100 µg/mL), alpha
Smooth Muscle Actin (SMA)(Dako/1A4/Mouse-IgG2a, (Leica/CD564/Mouse, 11 mg/L), CD16 (Cell Signaling/D1N9L/Rabbit lgG, 100 µg/mL), alpha
Smooth Muscle Actin (SMA)(Dako/1A4/Mouse-IgG2a, kappa 71 mg/L), Ki67 (Thermo
Fisher/SP6/Rabbit-IgG, 0.029 mg/ml), CD8 (Cell Signaling/ (Leica/CD664/Mouse-1964) and the Mouse-1964, Mouse-1964, Mouse 1964, Month Muscle Actin (SMA)(Dako/1A4/Mouse-1962a, kappa 71 mg/L), Ki67 (Thermo
Fisher/SP6/Rabbit-1gG, 0.029 mg/ml), CD8 (Cell Signaling/D1N9L/Rabbit IgG, 28 Fisher/SP6/Rabbit-IgG, 0.029 mg/ml), CD8 (Cell Signaling/D1N9L/Rabbit IgG, 28.5 mg/L), CD4
(Abcam/EPR6855/ Rabbit monoclonal-IgG, 100 µg/mL), PD1 (Abcam/EPR4877/ Rabbit
monoclonal-IgG, 1.85 mg/ml), CD20 (Leica/L26/Mouse-Ig Fisher/PR6855/ Rabbit monoclonal-IgG, 100 µg/mL), PD1 (Abcam/EPR4877/ Rabbit
Fisher/PA5-84069/ Polyclonal-Rabbit-IgG, 0.1 mg/mL), CD163 (Leica/10D6/Mouse, 49 mg/L).
Lot numbers are stated in Supplementary Table 1. Expressi (Abcam) 2002) Malan Manusuating (1997) 222 pagmany (222 pagmany 2002) (1912) Malan monoclonal-IgG, 1.85 mg/ml), CD20 (Leica/L26/Mouse-IgG2A, kappa, 95 mg/L), LAMP3 (Thermo Fisher/PA5-84069/ Polyclonal-Rabbit-IgG, 0.1 mg/mL Fisher/PA5-84069/ Polyclonal-Rabbit-IgG, 0.1 mg/mL), CD163 (Leica/10D6/Mouse, 49 mg/L).
Lot numbers are stated in Supplementary Table 1. Expression of cell markers was visualized
using Vector DAB (3,3'-diaminobenzidine) S Fig. 7.1 mumbers are stated in Supplementary Table 1. Expression of cell markers was visualized
using Vector DAB (3,3'-diaminobenzidine) Substrate kit (SK-4100). This methodology uses a
diaminobenzidine reaction to detect Lot numbers are stated in Supplementary Table 1. Expression of cell mathematic international
using Vector DAB (3,3'-diaminobenzidine) Substrate kit (SK-4100). This methodology uses a
diaminobenzidine reaction to detect ant using Vector DAB (3,3'-diaminobenzidine) Substrate kit (SK-4100). This methodology uses a
diaminobenzidine reaction to detect antibody labeling, together with a hematoxylin
counterstaining. A board-certified head and neck

diamino extended to detect and the computer and the transmit and the transmit processes controls for each marker.
 Spectral Library Creation. The spectral library was created according to Akoya Opal Assay

Development Gu controls for each marker.
 Spectral Library Creation. The spectral library was created according to Akoya Opal Assay

Development Guide (https://www.akoyabio.com/wp-content/uploads/2020/04/Opal-

Reagents_Brochure_Opal-A **Spectral Library Creatior**
Development Guide
Reagents Brochure Opal-
slides were made for ead
(DAPI), in addition to one
tested for sensitivity and Development Guide (https://www.akoyabio.com/wp-content/uploads/2020/04/Opal-
Reagents Brochure Opal-Assay-Development-Guide.pdf). Using HNSCC tissue, single-plex
slides were made for each marker with a matched fluorophore, Reagables were made for each marker with a matched fluorophore, 4',6-diamino-2-phenylindole
(DAPI), in addition to one unstained slide for autofluorescence detection. All antibodies were
tested for sensitivity and specific (DAPI), in addition to one unstained slide for autofluorescence detection. All antibodies were
tested for sensitivity and specificity to determine the optimal concentrations for primary
antibodies. Further optimization was (DAP), in addition to one unstanded and the automated concentration for automated for any detected for sensitivity and specificity to determine the optimal concentrations for primary antibodies. Further optimization was do tested for sensitivity and specificity to determine the optimal concentrations for primary
antibodies. Further optimization was done until spectral readouts for each marker were antibodies. Further optimization was done until spectral readouts for each marker were

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between 5-30 normalized counts (InForm, Akoya). In brief, representative areas from singleplex slaves are captured at 20x magnitication and the capture of the spectral library was assessed by using the R package <u>vignettes/unmixing quality report.Rmd</u> to measure unmixing quality. Raw images were spectrally unmi Ilbrary was assessed by using the R package <u>vignettes/unmixing quality report.Rmd</u> to
measure unmixing quality. Raw images were spectrally unmixed using the generated libraries
for later import into our data analysis plat

Multiplex Immunofluorescence (mIF) staining: We utilized a commercially available manual measure unmixing quality. Raw images were spectrally unmixed using the generated libraries
for later import into our data analysis platform (HALO v3.5.3577.173 Indica Labs).
Multiplex Immunofluorescence (mIF) staining: We measure unmining quality. Raw images mere spectrally annihiled using the generated libraries in the libraries
for later import into our data analysis platform (HALO v3.5.3577.173 Indica Labs).
Multiplex Immunofluorescence **Multiplex Immunofluorescence (mIF) staining:** We utilized a commercially ava
mIF staining kit (Opal 7-plex, Akoya, cat# NEL811001KT) and optimized the pr
markers and antibodies (Supp. Table S1). Briefly, serially cut sect multiplex immunohastescence (min) staining: We defined a commercially available manual
ministers and antibodies (Supp. Table S1). Briefly, serially cut sections were deparaffinized,
rehydrated, and subjected to heat-induce markers and antibodies (Supp. Table S1). Briefly, serially cut sections were deparaffinized,
rehydrated, and subjected to heat-induced epitope retrieval in Tris EDTA buffer pH 9.0 (Vector
Laboratories H-3301-250) or Citrat markers and antibodies (Supp. Table S1). Briefly, serially cut sections were deparatmized,
rehydrated, and subjected to heat-induced epitope retrieval in Tris EDTA buffer pH 9.0 (Vector
Laboratories H-3301-250) or Citratereflexation of two particles and sequential staining of each antibody was performed (exclusively for anti-CD20 antibody) and sequential staining of each antibody was performed until completion of two panels (one in each se (exclusively for anti-CD20 antibody) and sequential staining of each antibody was performed
until completion of two panels (one in each serially cut slide) per patient (Immune and TLS
panels). Nuclei were labeled using 47, (exclusively for anti-CD2 antibody) and sequential standing of each ministery for proteints

until completion of two panels (one in each serially cut slide) per patient (Immune and TLS

panels). Nuclei were labeled using 4 panels). Nuclei were labeled using 42,6-diamidino-2-phenylindole (DAPI, Spectral DAPI FP 1490,
Perkin Elmer, 1 ug/mL). Subsequently, a coverslip with mounting media (ProLong Glass Antifade
Mountant, P36980 inner, refractiv Perkin Elmer, 1 ug/mL). Subsequently, a coverslip with mounting media (ProLong Glass Antifade
Mountant, P36980 inner, refractive index 1.52, Invitrogen) was applied and cured for at least 6
hours.
Multispectral Imaging Pla

Mountant, P36980 inner, refractive index 1.52, Invitrogen) was applied and cured for at least 6
hours.
Multispectral Imaging Platform. All slides, including those stained using IHC approaches, were
imaged at 20X (NA 0.6, V Multispectral Imaging Platform. All slides, including those stained using IHC approaches, were
imaged at 20X (NA 0.6, Vectra3, Perkin Elmer, bulb intensity set to 10%). For each tissue
section, the entire tissue area was s Multis_|
imaged
section Imaged at 20X (NA 0.6, Vectra3, Perkin Elmer, bulb intensity set to 10%). For each tissue
section, the entire tissue area was scanned. Exposure times were as follows: Immune Panel:
exction, the entire tissue area was scann section, the entire tissue area was scanned. Exposure times were as follows: Immune Panel: section, the entire tissue area was scanned. Exposure times were as follows: Immune Panel: It is made available under a CC-BY-NC-ND 4.0 International license.

DOM THE IMAGE PROCESSING: 50 MS, CYS: 40.

DOM THE IMAGE PROCESSING: Following staining and imaging, serially cut tissue sections

Were registered, and a synchronous navigation tool was used to check fusion quality (HALO
 The Downstream mIF Image Processing: Followin

1905 were registered, and a synchronous navigat

193.5.3577.173 Indica Labs). Tumor-stroma c

193.5.3577.173 Indica Labs). Tumor-stroma c

193.5.3577.173 Indica Labs). Tumor-s Downstream min image Processing: Following staining and imaging, serially cut tissue sections
Were registered, and a synchronous navigation tool was used to check fusion quality (HALO
V3.5.3577.173 Indica Labs). Tumor-stro were registers, and a synchronous integrator tool was performed by a head and neck
pathologist (PS) using Hematoxylin Eosin (H&E) stained tumor sections. Following this process,
mIF-stained slides were classified using a r pathologist (PS) using Hematoxylin Eosin (H&E) stained tumor sections. Following this process,
mlF-stained slides were classified using a random forest classifier trained against the
pathologists' annotations. Single-cell mIF-stained slides were classified using a random forest classifier trained against the
pathologists' annotations. Single-cell phenotyping was done using Indica Labs – HighPlex FL
v4.1.3 module (Supp. Table S2). A detectab man states when there included any of the term there include a fighplex FL
pathologists' annotations. Single-cell phenotyping was done using Indica Labs – HighPlex FL
v4.1.3 module (Supp. Table S2). A detectable nucleus wa pathologists of the same thresholds were applied to all the slides, and spatial analysis was performed based on
average values.
Tertiary Lymphoid Structures quantification: Tertiary Lymphoid Structures were defined as
aggr

v4.1.3 module (Supp. Table S2). A detectable nucleus was manuatory to phemotype each cent.
The same thresholds were applied to all the slides, and spatial analysis was performed based on
average values.
Tertiary Lymphoid S Tertiary Lymphoid Structures quantification: Tertiary Lymphoid Structures were defined as
aggregates of lymphoid cells with histologic features resembling follicles in lymphoid tissue and
were identified on a serially cut Tertiary Lymph
aggregates of ly
were identified
identified on se
described above
(CD20+) and mo renary Lymphoid Structures quantification: Tertiary Lymphoid Structures were defined as
aggregates of lymphoid cells with histologic features resembling follicles in lymphoid tissue and
were identified on a serially cut sl so gate of lymphone cells in interesting of the limiting tend of lymphone and most
were identified on serially cut slides stained with mIF panels using random forest classifiers as
described above. At single-cell mIF phen dentified on serially cut slides stained with mIF panels using random forest classifiers as
described above. At single-cell mIF phenotyping, we included TLS with more than fifty B cells
(CD20+) and more than five dendriti described above. At single-cell mIF phenotyping, we included TLS with more than fifty B cells
(CD20+) and more than five dendritic cells (LAMP3+). The number of TLS was normalized by the
area analyzed (TLS/mm2). The averag (CD20+) and more than five dendritic cells (LAMP3+). The number of TLS was normalized by the
area analyzed (TLS/mm2). The average distance to the tumor area was calculated for all
patients. The peritumoral area was define area analyzed (TLS/mm2). The average distance to the tumor area was calculated for all
patients. The peritumoral area was defined as <100 µm from the tumor area (19)
Survival analysis: The Kaplan-Meier analysis was applied

patients. The peritumoral area was defined as <100 μ m from the tumor area (19)
Survival analysis: The Kaplan-Meier analysis was applied using the Log-rank test to assess the
association between the spatial location of patients. The Kaplan-Meier analysis was applied using the Log-rank test
association between the spatial location of TLS and both OS and PFS. Hazard random the tumor area (19) and the tumor area (19) $\frac{1}{2}$ Survival analysis: The Kaplan-Meier analysis was applied using the Log-rank test to assess the
association between the spatial location of TLS and both OS and PFS. Hazard ratios (HR) and association between the spatial location of TLS and both OS and PFS. Hazard ratios (HR) and

employed a bootstrapping approach to obtain more robust estimates of the hazard ratios and
their associated confidence intervals. Specifically, we performed 1,000 bootstrap resampling
iterations (R = 1000), where the orig their associated confidence intervals. Specifically, we performed 1,000 bootstrap resampling
iterations (R = 1000), where the original dataset was repeatedly resampled with replacement.
For each bootstrap replicate, the C therations (R = 1000), where the original dataset was repeatedly resampled with replacement.
For each bootstrap replicate, the Cox model was refit, and the hazard ratio was recalculated. All
values were considered statisti For each bootstrap replicate, the Cox model was refit, and the hazard ratio was recalculated. All
values were considered statistically significant if $a < 0.05$. Survival analyses were performed
with the survival (v3.5.5) For each bootstrap replies and statistically significant if a < 0.05. Survival analyses were performed
with the survival (v3.5.5) and survminer (v0.4.9) packages using R Statistical Software (v4.3.1, R
Core Team 2023).
Mac

with the survival (v3.5.5) and survminer (v0.4.9) packages using R Statistical Software (v4.3.1, R
Core Team 2023).
Machine learning model: To predict the response to ICB using spatial metrics, including TLS
features, we e Core Team 2023).

Machine learning model: To predict the response to ICB using spatial metrics, including TLS

features, we evaluated several multiclass classifiers, including Logistic Regression, Decision

Tree, ExtraTree Machine learning
features, we eval
Tree, ExtraTree, F
consisting of: 1. T
stroma (CD8 ratio Machine learning model: To predict the response to IcD damg spatial metrics, including 125
Features, we evaluated several multiclass classifiers, including Logistic Regression, Decision
Tree, ExtraTree, Random Forest, and Tree, ExtraTree, Random Forest, and Gradient Boosting. We used an initial set of features
consisting of: 1. The density of CD8+PD1+Ki67- in the tumor/density of CD8+PD1+Ki67- in the
stroma (CD8 ratio); 2. The density of CD Tree, Extrantant COS and CDS and COS+PD1+Ki67- in the tumor/density of CD8+PD1+Ki67- in the

Stroma (CDS ratio); 2. The density of CD20+ cells/density of CD163+ cells (CD20 ratio),

Combined Positive Score; 3. The average combined Positive Score; 3. The density of CD20+ cells/density of CD163+ cells (CD20 ratio),
Combined Positive Score; 3. The average size of TLS (bigger or smaller than the mean size in the
whole cohort); 4. TLS density (# Stroma (CD8 ratio); 2. The average size of TLS (bigger or smaller than the mean size in the
whole cohort); 4. TLS density (# of TLS/tissue size) via mIF. We also included spatial metrics
related to immune cell interactions

whole cohort); 4. TLS density (# of TLS/tissue size) via mlF. We also included spatial metrics
related to immune cell interactions.
Missing values were imputed using *Simplelmputer* from scikit-learn. Continuous features w which comomon cell interactions.

Missing values were imputed using *SimpleImputer* from scikit-learn. Continuous features were

then standardized using StandardScaler. We trained the models using a 75:25

training/validat Missing values were imputed using
then standardized using Standa
training/validation split, and a seare
select the set that resulted in the
variable, we visualized permutation Missing values were imputed using *Simple Imputer From seikk* learn. Continuous learnes were
then standardized using StandardScaler. We trained the models using a 75:25
training/validation split, and a search approach was training/validation split, and a search approach was used to test combinations of features to
select the set that resulted in the highest accuracy. To understand the contribution of each
variable, we visualized permutation select the set that resulted in the highest accuracy. To understand the contribution of each variable, we visualized permutation importance. We assessed the robustness of the results $\frac{d}{dt}$ select the set that resulted in the highest accuracy. To understand the contribution of each
variable, we visualized permutation importance. We assessed the robustness of the results variable, we visualize permutation importance. We assessed the robustness of the robustness of the results of
La problem in portance. We assessed the robustness of the results of the results of the robustness of the result

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through 5-fold cross-validated findings using a convolutional neural network
to predict ICB response directly from whole slide images. For this analysis, we preprocessed the
images by resizing them to 224x224 pixels and no In addition to classifier approach
to predict ICB response directly f
images by resizing them to 224x
augmented the image dataset us
generalization. We adopted a tra
a feature extractor. The base mc In a both to predict ICB response directly from whole slide images. For this analysis, we preprocessed the
Images by resizing them to 224x224 pixels and normalized pixel values to the range [0, 1]. We
augmented the image d to predict ICB response interesting and an interesting term in this analysis, the properties and images by resizing them to 224x224 pixels and normalized pixel values to the range [0, 1]. We augmented the image dataset usi augmented the image dataset using random rotations, shifts, flips, and zoom to improve model
generalization. We adopted a transfer learning approach using a pre-trained ResNet50 model as
a feature extractor. The base model and the image dataset using dimentions approach using a pre-trained ResNet50 model as
a feature extractor. The base model was modified by removing top layers and adding a custom
head consisting of a flattening layer, dense generalization. The base model was modified by removing top layers and adding a custom
head consisting of a flattening layer, dense layers, and dropout for regularization. The last 10
layers of the base model were fine-tun head consisting of a flattening layer, dense layers, and dropout for regularization. The last 10
layers of the base model were fine-tuned on our dataset. The model was trained using binary
cross-entropy loss and the Adam o head considers of the base model were fine-tuned on our dataset. The model was trained using binary
cross-entropy loss and the Adam optimizer. We implemented early stopping to prevent
overfitting and evaluated model perfor reposes and the Adam optimizer. We implemented early stopping to prevent
overfitting and evaluated model performance using 1,000 bootstrap resamples. This approach
allowed us to assess whether whole slide images, in combin overfitting and evaluated model performance using 1,000 bootstrap resamples. This approach
allowed us to assess whether whole slide images, in combination with extracted features, could
predict ICB response. To understand allowed us to assess whether whole slide images, in combination with extracted features, could predict ICB response. To understand features driving model predictions, we used Gradient-
weighted Class Activation Mapping (Gr predict ICB response. To understand features driving model predictions, we used Gradient-
weighted Class Activation Mapping (Grad-CAM) to visualize regions of the whole slide images
that were most influential in the model' weighted Class Activation Mapping (Grad-Cam) of Class Analysis Activation Mapping Attack Mapping Python v3.11.4, Jupyter notebook v6.5.4, scikit-learn v1.3.0, and TensorFlow v2.15.0.
Results:
Patient characteristics: Python v3.11.4, Jupyter notebook v6.5.4, scikit-learn v1.3.0, and TensorFlow v2.15.0.
Results:
Patient characteristics:

Results:

Pesults:
Patient characteristics:
Patient characteristics: Patient characteristics:

ratent clinicopathological features are stated in Table 1. The average age at the start of ICB
was 62 years old (range 50-81). The oral cavity was the most common primary site. High (220)
and moderate (1-19) CPS were obser and moderate (1-19) CPS were observed in most of the cohort. CPS did not differ between
responders and non-responders (*unpaired t-test, p= 0.3649*) (Supp. Figure 1. A). Fourteen
(70%) patients received pembrolizumab as m responders and non-responders (*unpaired t-test, p= 0.3649*) (Supp. Figure 1. A). Fourteen
(70%) patients received pembrolizumab as monotherapy, and six (30%) patients received ICB
plus chemotherapy.
Spatial Distribution

Spatial Distribution of Cells in the Tumor Microenvironment

(70%) patients received pembrolizumab as monotherapy, and six (30%) patients received ICB
plus chemotherapy.
Spatial Distribution of Cells in the Tumor Microenvironment
Multispectral profiling of individual cells within t plus chemotherapy.
 Spatial Distribution of Cells in the Tumor Microenvironment

Multispectral profiling of individual cells within tumor tissues allowed us to characterize the

distribution of 12 proteins within the tum put chemomently,
Spatial Distribution
Multispectral profilin
distribution of 12 p
found no difference
(unpaired t-test p=0 distribution of 12 proteins within the tumor microenvironment (TME) (Figure 1. A, B). We
found no difference in the area classified as tumor or stroma based on response to ICB
(*unpaired t-test p=0.6470, p=0.6108,* respec found no difference in the area classified as tumor or stroma based on response to ICB
(*unpaired t-test p=0.6470, p=0.6108,* respectively) (Figure 1. C). Cell distribution varied across
compartments in the TME. In the ar (*unpaired t-test p=0.6470, p=0.6108,* respectively) (**Figure 1. C**). Cell distribution varied across
compartments in the TME. In the area classified as tumor, the proliferation marker (Ki67+)
contributed to 35% and 36% o (unpaired t-test p=0.0470, p=0.0108, respectively) (Figure 1. C). Cell distribution varied across
compartments in the TME. In the area classified as tumor, the proliferation marker (Ki67+)
contributed to 35% and 36% of po contributed to 35% and 36% of positive cells for responders and non-responders, respectively.
Interestingly, immature B cells (CD19+ cells) contributed 10% to responders and 3% to non-
responders. The stromal area was char Interestingly, immature B cells (CD19+ cells) contributed 10% to responders and 3% to non-
responders. The stromal area was characterized by the presence of fibroblast (α-SMA) (26% in
responders and 20% in non-responders) responders and 20% in non-responders), followed by natural killer cells (CD16+) (15%) for
responders and CK+ cells for non-responders (19%) (Figure 1. C).
Spatial analysis identifies increased B cell density in responding

CK+ tumor cells or tumor area based on response status (Supp. Figure 1, 2; Supp. Table 3). responders and CK+ cells for non-responders (19%) (Figure 1. C).
Spatial analysis identifies increased B cell density in responding patients
We found no significant difference between the average distance of multiple cell responders and CK+ cens for non-responders (19%) (Figure 1. C).
Spatial analysis identifies increased B cell density in responding
We found no significant difference between the average distance
CK+ tumor cells or tumor ar Spatial analysis identifies increased B cell density in responding patients
We found no significant difference between the average distance of multipuck-
CK+ tumor cells or tumor area based on response status (Supp. Figure CK+ tumor cells or tumor area based on response status (Supp. Figure 1, 2; Supp. Table 3). CK+ tumor cells or tumor area based on response status (Supp. Figure 1, 2; Supp. Table 3).

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organization of dendritic cells (LAMP3+) in the TME. A trend toward an increased number of
exhausted T cells (CD8+PD1+Ki67-) within 20 um of dendritic cells (LAMP3+) was found in
responders compared to non-responders (*unp* exhausted T cells (CD8+PD1+Ki67-) within 20 um of dendritic cells (LAMP3+) was found in
responders compared to non-responders (*unpaired t-test p= 0.0576*) (Figure 2. A, B).
Additionally, a trend toward higher density of responders compared to non-responders (*unpaired t-test p= 0.0576*) (Figure 2. A, B).
Additionally, a trend toward higher density of proliferative dendritic cells (LAMP3+PD1-Ki67+)
was found in responders compared to non-Responders compared to non-responders (unpaired t-test $p= 0.0576$) (Figure 2. A, B).
Additionally, a trend toward higher density of proliferative dendritic cells (LAMP3+PD1-Ki67+)
was found in responders compared to non-Additionally, a significantly digiteration, or promotions (unpaired t-test $p = 0.22$) (Figure 2. C).
Interestingly, a significantly higher density of B cells (CD20+) was observed in responders
compared to non-responders (was found in responders compared to non-responders (unpaired t-test p= 0.22) (rigure 2. c).
Interestingly, a significantly higher density of B cells (CD20+) was observed in responders
compared to non-responders (unpaired t Compared to non-responders (unpaired *t-test p= 0.02*) (Figure 2. D, E). No other difference in
densities was found among groups (Supp. Figure 3; Supp. Table 4). The interplay of B cells with
other cells is critical for t compared to non-responders (unpaired t-test p= 0.02) (Figure 2. D, E). No other difference in
densities was found among groups (Supp. Figure 3; Supp. Table 4). The interplay of B cells with
other cells is critical for the

The organization of TLS within the TME impacts survival

other cells is critical for the development of TLS, leading us to investigate these structures in the
TME.
The organization of TLS within the TME impacts survival
The presence of TLS was assessed first on H&E slides by a h TME.
The organization of TLS within the TME impacts survival
The presence of TLS was assessed first on H&E slides by a head and neck pathologist (PS), who
provided a raw number of TLS per slide. Using their annotations an The o
The p
provid
minim
was s provided a raw number of TLS per slide. Using their annotations and setting a threshold for a
minimum of 5 dendritic cells (LAMP3+) and 50 B cells (CD20+) per structure, the presence of TLS
was subsequently assessed via mI provided a radio term in the provided and soles (CD20+) per structure, the presence of TLS
minimum of 5 dendritic cells (LAMP3+) and 50 B cells (CD20+) per structure, the presence of TLS
was subsequently assessed via mIF (was subsequently assessed via mIF (Figure 3. A). The detection rate of at least one TLS on the H&E slide by the pathologist was 50% (12/24) compared to 67% via mIF (16/24) for sequentially sectioned mIF slides. Importantly Was subsequently assessed via min (righte 5. A). The detection rate of at least one TLS on the
H&E slide by the pathologist was 50% (12/24) compared to 67% via mIF (16/24) for sequentially
sectioned mIF slides. Importantly Sectioned mIF slides. Importantly, a higher number of TLS was detected via mIF for all samples
except one (1408_ICI-25), for which the amount (one TLS) was the same for both approaches.
For the slides with replicates, a v except one (1408_ICI-25), for which the amount (one TLS) was the same for both approaches.
For the slides with replicates, a variation of <1 TLS was observed via pathologist assessment,
 $\frac{1}{2}$ For the slides with replicates, a variation of <1 TLS was observed via pathologist assessment,
for the slides with replicates, a variation of <1 TLS was observed via pathologist assessment, For the slides with replicates, a variation of \mathbb{R}^n term of \mathbb{R}^n assessment, assessment, assessment, and

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5).

The identification of TLS using mIF was positively correlated with the assessment done by the

pathologist on serially cut H&E sections $(R^2 = 0.66, p-value = <0.0001)$ (Figure 3.B). When

normalized by tissue size, res 5).
The
pat
nor
res
we
ran pathologist on serially cut H&E sections (R^2 = 0.66, p-value= <0.0001) (Figure 3.B). When
normalized by tissue size, responders tended to have higher TLS/mm² compared to non-
responders (*unpaired t-test*, p-value= 0 pathologist on serially cut H&E sections (R²
normalized by tissue size, responders tende
responders (*unpaired t-test, p-value= 0.0733*)
were constituted mainly by CD4+ T cells (21%
range 8-36) (Supp. Fig. 4. A; Supp. Ta = 0.00, p-value= \sim 0.0001) (Figure 3.B). When
d to have higher TLS/mm² compared to non-
(Figure 3. C) (Supp. Table 5). These structures
, range 14-40), followed by CD20+ B cells (17%,
o difference was found between th normalized by tissue size, responders tended to have higher TLS/mm⁻
responders (*unpaired t-test, p-value= 0.0733*) (Figure 3. C) (Supp. Table
were constituted mainly by CD4+ T cells (21%, range 14-40), followed by
range responders (*unpaired t-test, p-value*= 0.0733) (Figure 3. C) (Supp. Table 3). These structures
were constituted mainly by CD4+ T cells (21%, range 14-40), followed by CD20+ B cells (17%,
range 8-36) (Supp. Fig. 4. A; Supp range 8-36) (Supp. Fig. 4. A; Supp. Table 6). No difference was found between the distance of
TLS to tumor area among groups (Supp. Figure 4. B). Importantly, responders tended to have a
higher TLS area on average compared range 8-36) (Supp. Fig. 4. A; Supp. Faste 8). Instance was found between the distance of
TLS to tumor area among groups (Supp. Figure 4. B). Importantly, responders tended to have a
higher TLS area on average compared to n TES to tumor area among groups (Supp. Figure 4. B). Importantly, responders tended to have a
higher TLS area on average compared to non-responders (*unpaired t-test; p-value= 0.0932*)
(Figure 3. D), but there was no differ Figure 1.25 area on average compared to non-responders (*unpaired t-test; p-value= 0.0932)*
(Figure 3. D), but there was no difference in overall survival (OS) or progression-free survival
(PFS) between groups based on the (PFS) between groups based on the size of TLS (Supp. Figure 5. A-B). Among the 12 patients
with TLS, those whose average TLS distance to the tumor was within 100 µm had significantly
better outcomes. Specifically, the medi (PFS) between groups based on the size of TLS (Supp. Figure 3. A-B). Among the 12 patients
with TLS, those whose average TLS distance to the tumor was within 100 µm had significantly
better outcomes. Specifically, the med whetter outcomes. Specifically, the median OS was 33.5 months for patients with TLS within 100 μ m of the tumor area, compared to 11.6 months for those with a distance greater than 100 μ m (Log-rank p=0.05). Similarly, μ m of the tumor area, compared to 11.6 months for those with a distance greater than 100 μ m
(Log-rank p=0.05). Similarly, the median PFS was 26.3 months for patients with TLS within 100
 μ m of the tumor area versu (Log-rank p=0.05). Similarly, the median PFS was 26.3 months for patients with TLS within 100
 μ m of the tumor area versus 4.4 months for those in which the average TLS-tumor distance was

greater than 100 μ m (Log-ra (Log-rank period in which the average TLS-tumor distance was
greater than 100 μ m (Log-rank test p=0.03) (Figure 3. E-F).
A multivariable machine learning model allows for the prediction of response and survival
The res

A multivariable machine learning model allows for the prediction of response and survival

greater than 100 µm (Log-rank test p=0.03) (Figure 3. E-F).
A multivariable machine learning model allows for the prediction of response and survival
The response to ICB involves dynamic interactions between multiple comp A multivariable machine learning model allows for the pre
The response to ICB involves dynamic interactions bet
immune system and how those are organized in the TME. The response to ICB increases to ICB in the TME. Therefore, we decided to investigate
immune system and how those are organized in the TME. Therefore, we decided to investigate
the components in the TME. Therefore, we deci immune system and how those are organized in the TME. Therefore, we decided to investigate

between the ratio of exhausted T cells in the tumor to the stromal area (CD8+PD1+Ki67- density
in the tumor area/CD8+PD1+Ki67- density in the stroma) and clinical outcomes (Suppl. Fig 6. A-
C). Calculating the ratio of CD2 in the tumor area/CD8+PD1+Ki67- density in the stroma) and clinical outcomes (Suppl. Fig 6. A-
C). Calculating the ratio of CD20/CD163 densities, we observed a trend towards short OS (HR
0.43, 95% CI: 0.05-3.39; p-value= 0 in the tumor area/CD8+PD1+Ki67- density in the stroma) and climical outcomes (Suppl. Fig 6. A-
C). Calculating the ratio of CD20/CD163 densities, we observed a trend towards short OS (HR
0.43, 95% Cl: 0.05-3.39; p-value= 0 C). Calculating the ratio of CD20/CD103 densities, we observed a trend towards short OS (HR

0.43, 95% CI: 0.05-3.39; p-value= 0.4) and PFS (HR 0.3, 95% CI: 0.04-2.83; p-value= 0.3) for

patients whose ratio was below the

patients whose ratio was below the average for the cohort (<2.57), underscoring the potential
pro-tumoral potential of CD163+ cells (Suppl. Fig 7. A).
Spatial metrics were associated with response to ICB therapy. Natural pro-tumoral potential of CD163+ cells (Suppl. Fig 7. A).
Spatial metrics were associated with response to ICB therapy. Natural killer cells (CD16+Ki67-)
trended to be closer to proliferative dendritic cells (LAMP3+Ki67+) i Spatial metrics were associated with response to ICB t
trended to be closer to proliferative dendritic cells (LA
non-responders (p= 0.197) (Figure 4. A, B), and this av
correlated with response to ICB (p=0.098), with a hig Frended to be closer to proliferative dendritic cells (LAMP3+Ki67+) in responders compared to
non-responders (p= 0.197) (Figure 4. A, B), and this average distance trended to be negatively
correlated with response to ICB (the correlated with response to ICB (p=0.098), with a higher probability of response for patients
with shorter distances between these two cell populations. (Figure 4. C).
Subsequently, a multivariate model was used to pr

non-responders (p= 0.197) (Figure 4. A, B), and this average distance trended to be negatively
correlated with response to ICB (p=0.098), with a higher probability of response for patients
with shorter distances between th with shorter distances between these two cell populations. (Figure 4. C).
Subsequently, a multivariate model was used to predict the response of each patient based on
the spatial metrics. The best-performing model was the Subsequently, a multivariate model was used to predict the response of
the spatial metrics. The best-performing model was the K-Nearest Neig
achieved an accuracy of 0.80 (AUC 0.92) in predicting response, 1.00 (A
12-month Subsequently, a multimated model was the product to protect to protect to product the spatial metrics. The best-performing model was the K-Nearest Neighbors classifier, which achieved an accuracy of 0.80 (AUC 0.92) in pred achieved an accuracy of 0.80 (AUC 0.92) in predicting response, 1.00 (AUC 1.00) in predicting
12-month progression-free survival (PFS), and 0.80 (AUC 0.75) in predicting 12-month overall
survival (OS) (Figure 4. D). Featur 12-month progression-free survival (PFS), and 0.80 (AUC 0.75) in predicting 12-month overall
survival (OS) (Figure 4. D). Feature importance analysis demonstrated that the density of TLS
was the leading predictor consisten survival (OS) (Figure 4. D). Feature importance analysis demonstrated that the density of TLS
was the leading predictor consistently across all model architectures (Supp Figure 8). Other
important features in the models in survival (OS) (Figure 4. D). Feature importance analysis demonstrated that the density of TLS
was the leading predictor consistently across all model architectures (Supp Figure 8). Other
important features in the models in was the leading predictor consistently across all model architectures (Supp Figure 9). Other
important features in the models included the ratio of CD8+PD1+ in the tumor/stroma, the
CD20+/CD163+ ratio, and the LAMP3 stroma cD20+/CD163+ ratio, and the LAMP3 stromal density (Supp Figure 8). These findings support
CD20+/CD163+ ratio, and the LAMP3 stromal density (Supp Figure 8). These findings support CD20+/CD163+ ratio, and the LAMP3 stromal density (Supp Figure 8). These findings support

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determining the immune response and clinical outcomes in HNSCC patients treated with ICB.
A CNN was then trained to predict response directly from unannotated whole slide images
using the dataset of twenty patients. When c determining the dataset of twenty patients. When combined with the best-performing K-Neare
Neighbor model in an ensemble approach, the model achieved promising results in a small to
Neighbor model in an ensemble approach, using the dataset of twenty patients. When combined with the best-performing K-Nearest
Neighbor model in an ensemble approach, the model achieved promising results in a small test
set of five patients: an accuracy of 0.81 Neighbor model in an ensemble approach, the model achieved promising results in a small test
set of five patients: an accuracy of 0.81 (95% CI: 0.40 - 1.00) in predicting response, 1.00 (95%
CI: 0.40 - 1.00) in predicting Neighbor model in an ensemble approach, included achieved promining results in a small test
set of five patients: an accuracy of 0.81 (95% CI: 0.40 - 1.00) in predicting response, 1.00 (95%
CI: 0.40 - 1.00) in predicting o set of the patients an accuracy of the currents sately in prementing response) and (95% CI: 0.57 -
1.00) for one-year overall survival. To provide additional insight, a saliency map was generated
to visualize and interpret CI: 0.00) for one-year overall survival. To provide additional insight, a saliency map was generated
to visualize and interpret the features driving the model's predictions. This map offered insight
into the image characte 1.000) for visualize and interpret the features driving the model's predictions. This map offered insight
into the image characteristics, including Tertiary Lymphoid Structure-related features, that
appeared most influenti to visualize and interpret the features driving the model's predictions. This map offered insight
into the image characteristics, including Tertiary Lymphoid Structure-related features, that
appeared most influential in de into the image characteristics, including tertiary Lymphoid Structure related features, that
appeared most influential in determining patient outcomes (Figure 4. E, F).
Discussion
Here, we applied multi-spectral imaging me

Discussion

appeared most inhuential in determining patient outcomes (Figure 4. E, F).
Discussion
Here, we applied multi-spectral imaging methods to quantify twelve marke
tumor specimens, identified critical spatial signatures uniquel Here, we applied multi-personal imaging member to quantity in the matter in pre-ICB mitter
tumor specimens, identified critical spatial signatures uniquely found in patients with effective
responses to ICB, and differentia The specifical specified critical specified critical spatial signature differentially profile patients with a higher likelihood of res responses to ICB, and improved progression-free survival in patients whose average TLS epicenter-tumor
edge distance was within 100 μm, highlighting the importance of spatial localization in
biomarker studies. Additionall MENTAIN IMPROVER progression-free survival in patients investigating the special localization in
edge distance was within 100 µm, highlighting the importance of spatial localization in
biomarker studies. Additionally, with biomarker studies. Additionally, with a machine learning model consisting of spatial biology
metrics, together with raw image features, we were able to predict response and one-year
overall survival with 81% and 86% accura biomarker studies. An
interties, together with raw image features, we were able to predict response and one-year
overall survival with 81% and 86% accuracy, respectively. Importantly, TLS density was the
leading predictor means, together manimal analog features, the tree able to predict response and one-year
overall survival with 81% and 86% accuracy, respectively. Importantly, TLS density was the
leading predictor across models. CPS provid leading predictor across models. CPS provides a simple, single-marker view, whereas the leading predictor across models. CPS provides a simple, single-marker view, whereas the
control of the single-marker view, whereas the single-marker view, whereas the single-marker view, whereas the
control of the single-m

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that are important for response. The spatial metrics outperformed CPS, highlighting how
incorporating spatial biology can improve predictive models beyond conventional biomarkers.
Although CPS is used daily in the clinic,

incorporating spatial biology can improve predictive models beyond conventional biomarkers.
Although CPS is used daily in the clinic, its effectiveness as a predictive biomarker for ICB
response is limited. Patients with l although CPS is used daily in the clinic, its effectiveness as a predictive biomarker for IC
response is limited. Patients with low or negative CPS can still respond to ICB, while those wit
high CPS may not always experien Although CPS is limited. Patients with low or negative CPS can still respond to ICB, while those with
high CPS may not always experience a positive response (4,20–23) Additionally, using a single
marker to stratify a patie response is limited. Patients in the consisting of the can start response (4,20–23) Additionally, using a single
marker to stratify a patient's likelihood of response has limitations. First, whole tissue scoring
without di marker to stratify a patient's likelihood of response has limitations. First, whole tissue scoring
without discerning between tumor or stroma ignores the impact of cell localization within the
TME. Second, PD-L1 is express manned to strating between tumor or stroma ignores the impact of cell localization within the
TME. Second, PD-L1 is expressed in multiple cell phenotypes with various roles in response to
ICB. Therefore, PD-L1 positivity a TME. Second, PD-L1 is expressed in multiple cell phenotypes with various roles in response to
ICB. Therefore, PD-L1 positivity alone might not be enough to profile the TME, particularly
considering its variation across tim ICB. Therefore, PD-L1 positivity alone might not be enough to profile the TME, particularly
considering its variation across time (24). In our study, we seek to address these limitations by
using multi-step high-plex imagi ICB. THEREFORE, POSITIVE, THEREFORE, THEREFORE, PRINCIPS, particularly, particularly,

Considering its variation across time (24). In our study, we seek to address these limitations by

Using multi-step high-plex imaging t

consider multi-step high-plex imaging technology.
Dendritic cells are antigen-presenting cells and are responsible for priming CD4 and CD8 T cells
(25). Physical interactions between subsets of T cells and dendritic cells using main step high-presenting technology.
Dendritic cells are antigen-presenting cells and
(25). Physical interactions between subsets of
control antitumor immunity (26). Here, we sat (25). Physical interactions between subsets of T cells and dendritic cells have been shown to
control antitumor immunity (26). Here, we saw a trend toward increased average number of
exhausted T cells (CD8+PD1+Ki67-) withi (25). Physical interactions between subsets of T cells interactions between subsets of control antitumor immunity (26). Here, we saw a trend toward increased average number of exhausted T cells (CD8+PD1+Ki67-) within 20 µm exhausted T cells (CD8+PD1+Ki67-) within 20 μ m of dendritic cells (LAMP3+) in responders
compared to non-responders. This spatial localization might facilitate T cell priming by dendritic
cells in the context of ICB, r compared to non-responders. This spatial localization might facilitate T cell priming by dendritic
cells in the context of ICB, resulting in an improved antitumor response. LAMP3+ dendritic cells
have been shown to interac cells in the context of ICB, resulting in an improved antitumor response. LAMP3+ dendritic cells
have been shown to interact with neoantigen-reactive exhausted CD8 T cells and CD4 T regs in
cervical cancer, where LAMP3 was have been shown to interact with neoantigen-reactive exhausted CD8 T cells and CD4 T regs in
cervical cancer, where LAMP3 was associated with an immunosuppressive state, leading to
tumor escape and disease progression (27) cervical cancer, where LAMP3 was associated with an immunosuppressive state, leading to
tumor escape and disease progression (27). In contrast to our results, no statistical significance
tumor escape and disease progressio cervical cancer, where LAMP3 was associated with an immunity experience state, leading to
tumor escape and disease progression (27). In contrast to our results, no statistical significance tumor escape and disease progression (27). In contrast to our results, no statistical significance \mathbf{r}_i It is made available under a [CC-BY-NC-ND 4.0 International license](http://creativecommons.org/licenses/by-nc-nd/4.0/) .

was found in the density of dendritic cells (LAMP3+) among responders compared to non-

with more patients will be necessary to fully support these findings.

B cells produce antibodies specific to tumor-associated epitopes that can boost anti-tumor T

cell response and can act as antigen-presenting cells (28 B cells produce antibodies specific to tumor-associated epitopes t
cell response and can act as antigen-presenting cells (28). Tumor-ir
correlated with improved outcomes in multiple solid malignancie B cell response and can act as antigen-presenting cells (28). Tumor-infiltrating B cells have been
correlated with improved outcomes in multiple solid malignancies (29–32) and have been
shown to play a critical role in res correlated with improved outcomes in multiple solid malignancies (29–32) and have been
shown to play a critical role in response to ICB (10,11,33,34). Interestingly, higher densities of
CD20+ B cells and their physical int shown to play a critical role in response to ICB (10,11,33,34). Interestingly, higher densities of
CD20+ B cells and their physical interaction with CD8 T cells were associated with improved
prognosis in treatment-naive HP shown to play a critical role in response to ICE (25,23,23,2). Interestingly, higher densities of
CD20+ B cells and their physical interaction with CD8 T cells were associated with improved
prognosis in treatment-naive HPV prognosis in treatment-naive HPV+OPSCC patients despite analyzing only ten representative
low-powered visual fields per patient (35). Our results are aligned with prior studies and
support the anti-tumor role of B cells in prognosis in treatment (35). Our results are aligned with prior studies and
support the anti-tumor role of B cells in the TME (36,37).
The ratio of peri-tumoral CD8/FOXP3 densities is useful in detecting patients who are m

support the anti-tumor role of B cells in the TME (36,37).
The ratio of peri-tumoral CD8/FOXP3 densities is useful in detecting patients who are more
likely to respond to ICB in multiple solid malignancies (38–40). Here we support the ratio of peri-tumoral CD8/FOXP3 densities is useful
likely to respond to ICB in multiple solid malignancies (3.
survival outcomes for patients based on their tumor/s The ratio of peri-tumoral CD3/FOXP3 and alignancies (38–40). Here we found no difference in
Survival outcomes for patients based on their tumor/stroma ratio of exhausted CD8 T cell
densities (Supp. Fig 6). T cell exhaustio survival outcomes for patients based on their tumor/stroma ratio of exhausted CD8 T cell
densities (Supp. Fig 6). T cell exhaustion is a gradual process, with terminally exhausted T cells
being the less functional on the a survival outcomes for patients based on their child, process, with terminally exhausted T cells
being the less functional on the activation-exhaustion spectrum (41), a particular phenotype
that could have been missed by ou densities (Supp. Fig 6). Their exhaustion is a gradual process, with terminally exhausted T cells
being the less functional on the activation-exhaustion spectrum (41), a particular phenotype
that could have been missed by being that could have been missed by our 12 markers approach. A greater characterization of
exhaustion proteins could help further classify T cells and their response to ICB. Newer
technologies with increased capacity for exhaustion proteins could help further classify T cells and their response to ICB. Newer
technologies with increased capacity for the detection of spatial transcriptomic/proteomic data
(41,42) are paving the way for person echnologies with increased capacity for the detection of spatial transcriptomic/proteomic data
(41,42) are paving the way for personalized medicine and could provide greater insights into
the T cell – ICB interaction. (41,42) are paving the way for personalized medicine and could provide greater insights into
the T cell – ICB interaction. $(42,42)$ are paring the way for personalized medicine and could provide greater insights into the T cell – ICB interaction.

Innate and adaptive immune systems (9). They are characterized by a germinal center
composed of specialized immune cell types (42) and are created through the local
accumulation of CXCL13, RANKL, and interleukin-7 (43). TL composed of specialized immune cell types (42) and are created through the local
accumulation of CXCL13, RANKL, and interleukin-7 (43). TLS have been well-established as
positive prognostic factors in ICB (11,44). In HNSCC accumulation of *CXCL13, RANKL*, and interleukin-7 (43). TLS have been well-established as
positive prognostic factors in ICB (11,44). In HNSCC, the presence of TLS has been associated
with favorable outcomes and improved positive prognostic factors in ICB (11,44). In HNSCC, the presence of TLS has been associated
with favorable outcomes and improved responses to ICB regardless of HPV status (14,45). In
our study, the presence or absence of promote program that the model (11,45). In the presence of the presence of ICB regardless of HPV status (14,45). In
our study, the presence or absence of TLS was not associated with survival outcomes (Supp.
Fig. 9). Howeve our study, the presence or absence of TLS was not associated with survival outcomes (Supp.
Fig. 9). However, among patients with TLS, those with TLS located within 100 µm of the tumor
area showed significantly improved OS Fig. 9). However, among patients with TLS, those with TLS located within 100 µm of the tumor
area showed significantly improved OS and PFS (Figure 3). These findings align with prior
studies, which emphasize the importance Fig. 9). However, among patients with TLS, those with TLS located within 100 µm of the tumor
area showed significantly improved OS and PFS (Figure 3). These findings align with prior
studies, which emphasize the importance area showed significantly improved OS and PTS (Figure 3). These finalities align with prior
studies, which emphasize the importance of TLS spatial location over their mere presence (46).
However, reported peritumoral regio studies of TLS vary, ranging from 1000 µm to 5000 µm from
the tumor boundary (47). Larger studies are needed to reach a consensus on the predictive
value of TLS spatial organization. Through multivariate modeling, we ident However, reported peritumoral regions for TLS vary, ranging from 1000 pm from 1000 pm from 1000 pm
the tumor boundary (47). Larger studies are needed to reach a consensus on the predictive
value of TLS spatial organization The tumor boundary of TLS spatial organization. Through multivariate modeling, we identified TLS density as
the leading predictor of ICB response, outperforming conventional biomarkers such as CPS.
Notably, TLS can be meas value of the leading predictor of ICB response, outperforming conventional biomarkers such as CPS.
Notably, TLS can be measured in H&E slides, and recent research has demonstrated the
feasibility of automated computational The leading predictor of the liveponse, only the prediction of intertional biomatric such as the live
Notably, TLS can be measured in H&E slides, and recent research has demonstrated the
feasibility of automated computatio The measuring TLS density in lung
adenocarcinoma (48,49). This highlights the promise of TLS as a relatively easy-to-detect
biomarker with previously underestimated clinical significance. feasibility of automated computational reminisms for interesting TLS dentry in lung
adenocarcinoma (48,49). This highlights the promise of TLS as a relatively easy-to-detect
biomarker with previously underestimated clinica

aden arker with previously underestimated clinical significance.
Our study has limitations, including a small sample size and its retrospective design. As such,
these findings should be viewed as preliminary and indicative biomarker with the settimation of these findings should be viewed as preliminary and indicative or
these findings should be viewed as preliminary and indicative or
ther than definitive evidence of its efficacy. Additionall These findings should be viewed as preliminary and indicative of the potential of this approach

rather than definitive evidence of its efficacy. Additionally, 30% (6/20) of our cohort received

ICB in combination with che these finally shown as predicted as predictionally, and indicative of the potential of this approach.
The viewed as predicted in combination with chemotherapy. In these cases, the response observed in the four
ICB in combi ICB in combination with chemotherapy. In these cases, the response observed in the four
ICB in combination with chemotherapy. In these cases, the response observed in the four ICB in combination with chemotherapy. In these cases, the response observed in the four It is made available under a [CC-BY-NC-ND 4.0 International license](http://creativecommons.org/licenses/by-nc-nd/4.0/) .

promise as an easily identifiable biomarker that could be incorporated into the prognostic
workup for HNSCC. However, larger studies are needed to further assess the impact of TLS on
the response to ICB. provide for HNSCC. However, larger studies are needed to further assess the impact of TLS on
the response to ICB.
Conclusion working for HNSCC. However, larger studies are needed to further and impact of TLS on
the response to ICB.
We found that TLS density strongly predicts response to ICB therapy in HNSCC, outperforming

r
Conclusion
We found that TLS d
the current standar Conclusion
We found t
the current
integrated r We found that TLS dentity strongly predicts response to the unitity providers of the processfully
integrated multiple spatial metrics related to the organization and composition of immune cells
within the tumor microenviro integrated multiple spatial metrics related to the organization and composition of immune cells
within the tumor microenvironment. These findings show the importance of immune spatial
patterning in driving anti-tumor immun within the tumor microenvironment. These findings show the importance of immune spatial
patterning in driving anti-tumor immunity. They provide a rationale for developing therapies
promoting favorable TLS formation and com patterning in driving anti-tumor immunity. They provide a rationale for developing therapies
promoting favorable TLS formation and composition. Moreover, the results support the
incorporation of advanced multiplexed imagin promoting favorable TLS formation and composition. Moreover, the results support the
incorporation of advanced multiplexed imaging techniques that can measure the spatial
organization of the tumor microenvironment for futu promoting incorporation of advanced multiplexed imaging techniques that can measure the spatial
organization of the tumor microenvironment for future ICB predictive biomarker development.
Declaration of conflict of interes

organization of the tumor microenvironment for future ICB predictive biomarker development.
Declaration of conflict of interest (COI): The authors have no COI related to this work to declare. organization of conflict of interest (COI): The authors have no COI related to this work to
declare.
Author contributions: S.L.S., D.L. F., and M.S.F. conceptualized the investigation and provided

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Author contributions: S.L.S., D.L. F., and M.S.F. conceptualized the investigation and provided
supervision. D.R.T, S.H., **Author c**
supervisi
M.E.B, a Author contributions: S.L.S., D.L. F., and M.S.F. conceptualized the investigation and provided
supervision. D.R.T, S.H., and E.L. performed sample processing, staining, and scanning. D.A.R.T
M.E.B, and P.S. performed form M.E.B, and P.S. performed formal analysis, including visualization. R.M., T.R., M.P., J.C.P., and
L.J.W. assisted with study design and sample acquisition. D.A.R.T. wrote the original draft, and
all authors performed criti M.E.B, and P.D. performed Fridman analysis, including visualization. Individually, the system, and
L.J.W. assisted with study design and sample acquisition. D.A.R.T. wrote the original draft, an
all authors performed criti all authors performed critical reviews and revisions.

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Istari Oncology and served as a consultant for Galvanize Therapeutics. Mostari Oncology and served as a consultant for Galvanize Therapeutics.
Istari Oncology and served as a consultant for Galvanize Therapeutics. Istari Oncology and served as a consultant for Galvanize Therapeutics.

Figure legends

Figure 1. Study workflow and characterization of the tumor-immune microenvironment in Image analysis using a multi-step pipeline including machine learning algorithms. Sequentially
cut slides were stained with different staining panels, scanned, and fused using our image
analysis platform (HALO, Indica Labs cut slides were stained with different staining panels, scanned, and fused using our image
analysis platform (HALO, Indica Labs). **1 B.** Multi-immunofluorescence staining was performed;
a representative image from patient cut since were staining minimalized stating panels, stating was managed an analysis platform (HALO, Indica Labs). 1 B. Multi-immunofluorescence staining was performed;
a representative image from patient S-69_M3 is display a representative image from patient S-69_M3 is displayed here. All markers are shown (left),
and a single marker view (right) is shown. 1 C. Each marker's contribution percentage to the
tumor area (1) and stroma (2) is sho and a single marker view (right) is shown. **1 C.** Each marker's contribution percentage to the tumor area (1) and stroma (2) is shown based on response to ICB. Additionally, differences between the tumor and stroma areas and a single marker view (right) is shown. **1 C.** Each marker's contribution percentage to the
tumor area (1) and stroma (2) is shown based on response to ICB. Additionally, differences
between the tumor and stroma areas b the tumor and stroma areas based on response to ICB are shown (3,4).
Between the tumor and stroma areas based on response to ICB are shown (3,4). between the tumor and stroma areas based on response to ICB are shown (3,4).

Figure 2. Spatial analysis of HNSCC TME reveals preferential organization of antigenpresenting cens. 2 A. A representative image of T cell (CD8+PD1+Ki67-) organization within 20

4 proximity analysis between T

cells and dendritic cells is shown (right). 2 B. Average number of T exhausted T cells

(CD8+PD μ m of a dendritic cells is shown (right). **2 B.** Average number of T exhausted T cells
(CD8+PD1+Ki67-) within 20 μ m of dendritic cells (LAMP3+) is shown. **2 C.** A rug plot displays the
proliferative dendritic cell ((CD8+PD1+Ki67-) within 20 µm of dendritic cells (LAMP3+) is shown. **2 C.** A rug plot displays the
proliferative dendritic cell (LAMP3+PD1-Ki67+) densities based on response to ICB. **2 D.** B cell
density heatmap among one r (CD8+1 D1+Ki67-) within 20 µm of dentative cens (CAMF3+1) is shown. 2 C. A rug plot displays the
proliferative dendritic cell (LAMP3+PD1-Ki67+) densities based on response to ICB. 2 D. B cell
density heatmap among one resp promerative dendritic cell (LAMP3+PD1-Ki67+) densities based on response to ICB. 2 D. B cell
density heatmap among one responder (ICI-11) and one non-responder (ICI-26). 2 D. A rug plot
shows the densities of B cells (CD20

density heatmap among one responder (ICI-11) and one non-responder (ICI-20). 2 D. A rug plot
shows the densities of B cells (CD20+) based on response to ICB.
Figure 3. The spatial organization of tertiary lymphoid structur Figure 3. The spatial organization of tertiary lymphoid struct
closer view of a TLS is displayed using high-plex immunofluoresco
of each marker to the TLS (right). 3 B. Correlation between mll Figure 3. The spatial organization of tertiary lymphoid structures impacts survival. 3 A. A.
Closer view of a TLS is displayed using high-plex immunofluorescence (left) and the contribution
of each marker to the TLS (right of each marker to the TLS (right). **3 B.** Correlation between mIF and pathologist in identifying
TLS in serially cut slides. **3 C.** Number of TLS normalized by the tissue size. **3 D.** Average TLS
area in responders compare TLS in serially cut slides. **3 C.** Number of TLS normalized by the tissue size. **3 D.** Average TLS area in responders compared to non-responders. **3 E, F.** Overall and progression-free survival based on the average distanc TLS in serially cut slides. 3 C. Number of TLS normalized by the tissue size. 3 D. Average TLS
area in responders compared to non-responders. 3 E, F. Overall and progression-free survival
based on the average distance of T

area in responders compared to non-responders. **3 L, F.** Overall and progression rice survival
based on the average distance of TLS to the tumor area.
Figure 4. A multivariable machine learning model allows for the predict Figure 4. A multivariable machine learning model allow
and survival in HNSCC. 4 A. Comparison between tl
(CD16+Ki67-) to proliferative dendritic cells (LAMP3+Ki6) Figure 4. A multivariable machine learning model allows for the prediction of response to ICB (CD16+Ki67-) to proliferative dendritic cells (LAMP3+Ki67+) based on the response to ICB. 4 B.
Representative image from the analysis in 4 A.,4 C. Correlation between the average distance of
natural killers (CD16+Ki67-) to Representative image from the analysis in 4 A.,4 C. Correlation between the average distance of
Representative image from the analysis in 4 A.,4 C. Correlation between the average distance of
natural killers (CD16+Ki67-) t Representative image from the analysis in 4 A.,4 C. Correlation between the average distance of
natural killers (CD16+Ki67-) to proliferative dendritic cells (LAMP3+Ki67+) and response to ICB.
4 D. Model performance: Area 4 D. Model performance: Area under the curve (AUC) for the models used to predict response
to ICB. 4 E. Convolutional Neural Network workflow. (A) Original Image: Cross-sectional medical
to ICB. 4 E. Convolutional Neural N 4 D. Model performance: Area under the curve (AUC) for the models used to predict response
to ICB. 4 E. Convolutional Neural Network workflow. (A) Original Image: Cross-sectional medical
to ICB. 4 E. Convolutional Neural N to ICB. 4 E. Convolutional Neural Network workflow. (A) Original Image: Cross-sectional medical

image classification. Brighter areas indicate higher importance, and darker areas (blue/green)
indicate less importance. (C) Superimposed Image: Original image overlaid with Grad-CAM++
heatmap, highlighting model-focused a indicate less importance. (C) Superimposed Image: Original image overlaid with Grad-CAM++
heatmap, highlighting model-focused areas within tissue context. 4 F. Layer Activations: Four
feature maps from the initial convolut inariate less importance. (C) Superimposed image: Original image overlain with State Line
heatmap, highlighting model-focused areas within tissue context. 4 F. Layer Activations: Four
feature maps from the initial convolut heatmap, mgmighting model-focused areas within tissue context. 4 F. Layer Activations: Four
feature maps from the initial convolutional layer of the ResNet model. Each panel represents a
distinct activation channel, reveal feature maps from the initial convolutional layer of the ResNet Model. Each pairs represents a
distinct activation channel, revealing different low-level features extracted by the network.
Supplementary Figure 1. A. CPS sc

Supplementary Figure Legends

distinct activation channel, revealing and channel features extracted by the network.
Supplementary Figure 1. A. CPS score based on response. B. Average distance of each ma
to CK+ cell. C. Average distance of key markers t

Supplementary Figure 1. A. CPS score based on response. B. Average distance of each marker
to CK+ cell. C. Average distance of key markers to proliferative dendritic cells (LAMP3).
Supplementary Figure 2. A. Average distan to CH+ cell. COMP.
Supplementary Figure 2. A. Average distance to tumor/stroma interface. B. Distance
Supplementary Figure 3. Densities among responders and non-responders. Supplementary Figure 2. A. Average distance to tumor/stroma interface. B. Bistance to tumor
area.
Supplementary Figure 4. TLS characterization. A. Contribution of each marker to TLS. B.

area.
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Avera Supplementary Figure 3. Densities among responders and non-responders.
Supplementary Figure 4. TLS characterization. A. Contribution of each
Average distance of TLS to tumor area.
Supplementary Figure 5. Survival outcomes Supplementary Figure 4. TLS characterization. A. Contribution of each marker to TLS. B.
Average distance of TLS to tumor area.
Supplementary Figure 5. Survival outcomes based on average TLS size
Supplementary Figure 6. Tum

Mongo allamed in the taming area.
Supplementary Figure 5. Survival outco
Supplementary Figure 6. Tumor/strom
and survival outcomes are based on h Supplementary Figure 5. Survival outcomes based on average TLS size
Supplementary Figure 6. Tumor/stroma ratio of densities of exhausted
and survival outcomes are based on higher or lower than the mean
Overall survival for Supplementary Figure 6. Tumor/stroma ratio of densities of exhausted T cells (CD8+PD1+Ki67-)
and survival outcomes are based on higher or lower than the mean value for the cohort. A.
Overall survival for the ratio of exhau Overall survival for the ratio of exhausted T cells in the tumor/stroma. B. Progression-free

Decrall survival for the ratio of exhausted T cells in the tumor/stroma. B. Progression-free

Letter in the tumor/stroma. B. Pr Overall survival for the ratio of exhausted T cells in the tumor/stroma. B. Progression-free It is made available under a CC-BY-NC-ND 4.0 International license.

showing a strong presence of exhausted T cells in the tumor.
 Supplementary Figure 7. The ratio of CD20/CD163 densities for the whole slide and survival

outcomes are based on higher or lower than the mean value for the Supplementary Figure 7. The ratio of CD20/CD163 densitie
outcomes are based on higher or lower than the mean value
and B. progression-free survival. C. Representative image of (Supplementary Figure 7. The ratio of CD20/CD163 densities for the whole slide and survival
outcomes are based on higher or lower than the mean value for the cohort. A. Overall survival
and B. progression-free survival. C. out come in the based on higher or lower than the mean value for the contract or lower than values.
and B. progression-free survival. C. Representative image of CD20 and CD163 cells in the tumor
microenvironment of a respo

and B. progression-free survival. C. Representative image of CD20 and CD163 cm and CD163 cells in the tumor
microenvironment of a responder to ICB.
Supplementary Figure 8. Feature Importance for Multiclass Classifiers. TLS Supplementary Figure 8. Feature Importa
TLS via mIF; TLS area (mm²): TLS den:
CD8+PD1+Ki67-_20µm_DC: average quar Supplementary Figure 8. Feature Importance for Multiclass Classifiers. TLS/µm (imit): density of
TLS via mlF; TLS area (mm²): TLS density using raw counts assessed by the pathologist.
CD8+PD1+Ki67-_20µm_DC: average quant TLS via miF; TLS area (mm
CD8+PD1+Ki67-_20µm_DC: av
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Ki67+: Average distance of pr
Average distance of CD16+Ki6 erage quantity of exhausted T cells in 20 µm radius distance of
RATIO: ratio of CD8+PD1+Ki67+ in tumor/stroma. IA_CD8+PD1-
roliferative CD8+ T cells to the tumor area. CD16+Ki67- to PDC:
57- cells to proliferative dendriti dendritic cells (LAMP3). CD8RATIO: ratio of CD8+PD1+Ki67+ in tumor/stroma. IA_CD8+PD1-
Ki67+: Average distance of proliferative CD8+ T cells to the tumor area. CD16+Ki67- to PDC:
Average distance of CD16+Ki67- cells to pro Ki67+: Average distance of proliferative CD8+ T cells to the tumor area. CD16+Ki67- to PDC:
Average distance of CD16+Ki67- cells to proliferative dendritic cells (LAMP3+Ki67+). TLS_size:
raw average size of TLS. LAMP3STROM Average distance of CD16+Ki67- cells to proliferative dendritic cells (LAMP3+Ki67+). TLS_size:
raw average size of TLS. LAMP3STROMA: Density of dendritic cells (LAMP3+) in stroma area.
CPS: Combined positive score. CD20RAT Average size of TLS. LAMP3STROMA: Density of dendritic cells (LAMP3+) in stroma area.
CPS: Combined positive score. CD20RATIO: Ratio of CD20/CD163 densities in the whole tissues
section. raw average size of TLS. LAMP3 CHAMP3 STROMATIO: Ratio of CD20/CD163 densities in the whole tissues
Section.
Supplementary Figure 9. Presence or absence of TLS. A. Overall survival. B. Progression-free.

Section.
Supplementary Figure 9. Presence or absence of TLS. A. Overall survival. B. Progression-free
survival. Supplen
survival.
. Supplementary Figure 9. Presence or absence of TLS. A. Overall survival. B. Progression-free
survival.

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1 B. Multi-immunofluorescence staining: representative image from one responder to ICB (S-69_M3). 13 marker image highlighting the presence of a Tertiary Lymphoid Structure (left) . Single marker image at high magnification (right)

1 A. Study workflow: Tumor specimen retrieval, staining, scanning, and image analysis using a multi-step pipeline.

Figure 1. Study workflow and characterization of the tumor-immune microenvironment in HSNCC

Each marker's contribution to

Each marker's contribution to

Figure 2. Spatial analysis of HNSCC TME reveals preferential organization of antigen-presenting cells.

2 A. Representative image of T cell (CD8+PD1+Ki67-))organization within 20 µm of a dendritic cell (LAMP3+) in a responder to ICB (ICI-11).

2 B. Exhausted T cells (CD8+PD1+Ki67-) within 20 µm of dendritic cells (LAMP3+ cells)

2 D. B cell density heatmap among one responder (ICI-11) and one non-responder (ICI-26)

2 C. Proliferative dendritic cell (LAMP3+PD1-Ki67+) densities based on response to ICB

Value

Figure 3. The spatial localization of tertiary lymphoid structures impacts survival

3 B. Identification of TLS: Correlation between mIF and pathologist

3 A. Closer view of a TLS using the high-plex panel (left) and a closer view of markers with highest contribution to the TLS (right).

3 E. OS: TLS Distance to Tumor

Figure 4. A multivariable machine learning model allows for the prediction of response to ICB and survival in HNSCC

4 B. Representative image from a responder displays proximity analysis between CD16+Ki67- cells and LAMP3+Ki67+ cells

proliferative dendritic cells (LAMP3+KI67+) $\begin{array}{lll} {\small\texttt{CD16+K167-to PDC}}\ {\small\texttt{8}}\ {\small\texttt{8}}\ {\small\texttt{8}}\ {\small\texttt{8}}\ \end{array}$ 400 200 **Non- Responder Responder** CD16+Ki67- to PDC (Scaled) **4 C.** Correlation between Logistic Regression Coef: -0.602 P=0.098 Logistic Regression
Coef: -0.602 the average distance of medRxiv preprint doi: [https://doi.org/10.1101/2024.09.10.24313432;](https://doi.org/10.1101/2024.09.10.24313432) this version posted September 12, 2024. The copyrigh holder for this
CD16Th Mas and the author/funder, who has granted medRxiv a license to display the pre It is made available under a CC-BY-NC-ND 4.0 International license proliferative dendritic cells (LAMP3+KI67+) and $\frac{w}{r}$ 0.3 response to ICB Probabilit 0.2 0.1 0.0 -0.5 0.0 0.5 1.0 2.0 2.5 3.0 1.5 CD16+Ki67- to PDC_scaled

1000

4 E. Convolutional Neural Network workflow

CD16+Ki67- to PDC Mann Whitney U: 32.0 P=0.197

4 D. Model performance

4 A. Average distance of

CD16+Ki67- cells to

4 F. Layer activations and features extracted from the network

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Table 1. Clinical characteristics of the cohort

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