Supplementary material

Computational image analysis of T-cell infiltrates in resectable gastric cancer: association with survival and molecular subtypes

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**Supplementary Table 1.** Primary antibody and associated fluorophore application; supplier and dilution.

| Primary antibody | Supplier        | Dilution | Fluorophore (PerkinElmer) | Dilution |
|------------------|-----------------|----------|---------------------------|----------|
| Anti-CD4         | PerkinElmer     | 1:100    | Opal 520                  | 1:100    |
| Anti-CD8         | PerkinElmer     | 1:300    | Opal 570                  | 1:150    |
| Anti-FoxP3       | PerkinElmer     | 1:750    | Opal 620                  | 1:150    |
| Anti-CD45RO      | PerkinElmer     | 1:300    | Opal 650                  | 1:150    |
| Anti-pan cytokeratin (AE1/AE3) | Dako   | 1:500    | Opal 690                  | 1:150    |
Supplementary Table 2. Vectra 3.0 scanning exposure times

| Counterstain or primary antibody | Fluorophore | Filter  | 10x overview exposure time (ms) | 20x scanning exposure time (ms) |
|----------------------------------|-------------|---------|---------------------------------|----------------------------------|
| Spectral DAPI                    | N/A         | DAPI    | 5                               | 40                               |
| Anti-CD4                         | Opal 520    | FITC    | 40                              | 150                              |
| Anti-CD8                         | Opal 570    | Cy3     | 40                              | 150                              |
| Anti-FoxP3                       | Opal 620    | Texas Red | 40               | 150                              |
| Anti-CD45RO                      | Opal 650    | Cy5     | 40                              | 150                              |
| Anti-pan cytokeratin (AE1/AE3)   | Opal 690    | Cy5     | 40                              | 150                              |
Supplementary Table 3. HALO algorithm setting for the nuclear, CD4-cell, CD8-cell, CD45RO-cell, FOXP3-cell and cytokeratin multicolour immunofluorescence computational detection.

| Analysis magnification | Setting |
|------------------------|---------|
| Image zoom             | 1       |

**Nuclear detection**

| Setting                                    |         |
|--------------------------------------------|---------|
| Nuclear contrast threshold                 | 0.5     |
| Minimum nuclear intensity                  | 4       |
| Nuclear segmentation aggressiveness        | 0.7     |
| Fill nuclear holes                         | False   |
| Nuclear size                              | 9,700   |
| Minimum nuclear roundness                 | 0       |
| Opal 520 (CD4) weight                     | 0       |
| Opal 570 (CD8) weight                     | 0       |
| Opal 620 (FOXP3) weight                   | 1       |
| Opal 670 (CD45RO) weight                  | 0       |
| Opal 690 (Pancytokeratin) weight           | 0       |
| DAPI weight                                | 1       |
| Autofluorescence weight                    | 0       |

**Membrane and Cytoplasmic detection**

| Setting                                    |         |
|--------------------------------------------|---------|
| Maximum cytoplasmic radius                 | 0.6     |
| Membrane segmentation aggressiveness       | N/A     |
| Cell size                                  | 6,500   |
| Opal 520 to Opal 690 membrane segmentation| False   |
| DAPI membrane segmentation                 | False   |
| Autofluorescence membrane segmentation     | False   |

**Opal 520 (CD4)**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 0.4     |
| Cytoplasm positive threshold  | 0.3     |
| Membrane positive threshold   | 7       |

**Opal 570 (CD8)**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 1.4     |
| Cytoplasm positive threshold  | 1.2     |
| Membrane positive threshold   | 94      |

**Opal 620 (FOXP3)**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 3       |
| Cytoplasm positive threshold  | 89      |
| Membrane positive threshold   | 89      |

**Opal 670 (CD45RO)**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 1       |
| Cytoplasm positive threshold  | 1       |
| Membrane positive threshold   | 34      |

**Opal 690 (Pan cytokeratin)**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 0.4     |
| Cytoplasm positive threshold  | 1       |
| Membrane positive threshold   | 21      |

**DAPI**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 0       |
| Cytoplasm positive threshold  | 70      |
| Membrane positive threshold   | 70      |

**Autofluorescence**

| Setting                        |         |
|-------------------------------|---------|
| Nucleus positive threshold    | 26      |
| Cytoplasm positive threshold  | 26      |
| Membrane positive threshold   | 26      |
**Supplementary Table 4.** QuPath algorithm settings for chromogenic nuclear FOXP3 stain computational detection. 0 and 1+ detections were defined as FOXP3 negative, 2+ and 3+ detections were defined as FOXP3 positive.

| Setup parameters                      | Setting                          |
|--------------------------------------|----------------------------------|
| Detection image                      | Optical density sum              |
| Requested pixel size                 | 0.5μm                            |
| **Nucleus parameters**               |                                  |
| Background radius                    | 8.0μm                            |
| Median filter radius                 | 0.0μm                            |
| Sigma                                | 1.5μm                            |
| Minimum area                         | 10.0μm²                          |
| Maximum area                         | 400.0μm²                         |
| Threshold                            | 0.1                              |
| Maximum background intensity         | 2.0                              |
| Split by shape                       | Checked                          |
| Exclude DAB (membrane staining)      | Checked                          |
| **Cell parameters**                  |                                  |
| Cell expansion                       | 5μm                              |
| Include cell nucleus                 | Checked                          |
| **General parameters**               |                                  |
| Smooth boundaries                    | Checked                          |
| Make measurements                   | Checked                          |
| **Intensity threshold parameters**   |                                  |
| Score compartment                    | Nucleus: DAB OD mean             |
| Threshold 1+                         | 0.10                            |
| Threshold 2+                         | 0.30                            |
| Threshold 3+                         | 0.60                            |
| Single threshold                     | Unchecked                        |
Supplementary Table 5. Multivariable Cox regression analysis with backwards Wald selection of non-significant variables within the discovery cohort.

| Variable                | Initial analysis – Step 1 | Step 2 | Step 3 | Final analysis – Step 4 |
|-------------------------|---------------------------|--------|--------|-------------------------|
|                         | Hazard ratio (95% CI)     | P value* | Hazard ratio (95% CI) | P value* | Hazard ratio (95% CI) | P value* |
| CD45RO-cell density     |                           |        |        |                         |
| Hi                      | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | 1.00 (Reference) | -      |
| Int                     | 1.70 (1.09 to 2.64)       | 0.02   | 1.69 (1.09 to 2.62) | 0.02   | 1.72 (1.11 to 2.66) | 0.02   | 1.71 (1.11 to 2.65) | 0.02   |
| Lo                      | 2.03 (1.21 to 3.41)       | 0.007  | 2.02 (1.21 to 3.39) | 0.007  | 2.05 (1.23 to 3.42) | 0.006  | 2.09 (1.25 to 3.48) | 0.005  |
| FOXP3-cell density      |                           |        |        |                         |
| Hi                      | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | 1.00 (Reference) | -      |
| Int                     | 2.02 (1.21 to 3.35)       | 0.007  | 2.00 (1.21 to 3.30) | 0.007  | 1.98 (1.20 to 3.26) | 0.007  | 2.00 (1.21 to 3.29) | 0.007  |
| Lo                      | 2.84 (1.55 to 5.22)       | 0.001  | 2.81 (1.55 to 5.12) | 0.001  | 2.78 (1.53 to 5.05) | 0.001  | 2.79 (1.54 to 5.08) | 0.001  |
| Stage (UICC TNM 7th Edition) |                         |        |        |                         |
| pT3/4                   | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | 1.00 (Reference) | -      |
| pT1/2                   | 0.21 (0.10 to 0.45)       | <0.001 | 0.21 (0.10 to 0.45) | <0.001 | 0.21 (0.10 to 0.43) | <0.001 | 0.22 (0.10 to 0.45) | <0.001 |
| pN1-3                   | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | 1.00 (Reference) | -      |
| pN0                     | 0.47 (0.30 to 0.73)       | 0.001  | 0.47 (0.30 to 0.74) | 0.001  | 0.47 (0.30 to 0.72) | 0.001  | 0.45 (0.29 to 0.70) | <0.001 |
| MMR status              |                           |        |        |                         |
| MMRp                    | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | -          | -      |
| MMRd                    | 0.66 (0.35 to 1.22)       | 0.18   | 0.65 (0.35 to 1.22) | 0.18   | 0.66 (0.36 to 1.23) | 0.19   | -          | -      |
| Lauren classification    |                           |        |        |                         |
| Intestinal              | 1.00 (Reference)          | -      | 1.00 (Reference) | -      | -          | -      |
| Diffuse/Mixed           | 0.84 (0.61 to 1.1)        | 0.29   | 0.84 (0.61 to 1.16) | 0.28   | -          | -      |
| EBV status              |                           |        |        |                         |
| EBV-                    | 1.00 (Reference)          | -      | -      | -          | -      |
| EBV+                    | 1.13 (0.35 to 3.69)       | 0.84   | -      | -          | -      |

*Two-sided, Cox regression analysis.
**Supplementary Figure 1.** Cancer specific survival in the discovery and validation cohorts. Dashed lines indicate the median survival time for each cohort.
Supplementary Figure 2. Cancer specific survival in EBV+, MMRd and MMRp/EBV- groups across the discovery and validation cohorts combined. Dashed lines indicate the median survival time for each group, where this is reached.
**Supplementary Figure 3.** Median CD8-cell, CD45RO-cell and FOXP3-cell densities in the discovery and validation cohorts. Densities were off-set by 1 before log transformation. Red bars indicate the median and green whiskers the interquartile range.