Single-cell tumor-immune microenvironment of BRCA1/2 mutated high-grade serous ovarian cancer

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1 **Single-cell tumor-immune microenvironment of BRCA1/2 mutated high-grade serous ovarian cancer**

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37 **Abstract**

38 The majority of high-grade serous ovarian cancers (HGSCs) are deficient in homologous recombination (HR)
39 DNA repair, most commonly due to mutations or hypermethylation of the *BRCA1/2* genes. We aimed to
40 discover how *BRCA1/2* mutations shape the cellular phenotypes and spatial interactions of the tumor
41 microenvironment.

42 Using a highly multiplex immunofluorescence and image analysis we generated spatial proteomic data for 21
43 markers in 124,623 single cells from 112 tumor cores originating from 31 tumors with *BRCA1/2* mutation
44 (*BRCA1/2*mut), and from 13 tumors without alterations in HR genes (HRwt). We identified a phenotypically
45 distinct tumor microenvironment in the *BRCA1/2*mut tumors with evidence of increased immunosurveillance.
46 Importantly, we found an opposing prognostic role of a proliferative tumor-cell phenotypic subpopulation in
47 the HR-genotypes, which associated with enhanced spatial tumor-immune interactions by the CD8+ and
48 CD4+T-cells in *BRCA1/2*mut tumors. The single-cell spatial landscapes indicate distinct patterns of spatial
49 immunosurveillance with the premise to improve immunotherapeutic strategies and patient stratification in
50 HGSC.

51

52 **Introduction**

53 Every year almost 240 000 new cases of ovarian cancers are diagnosed, leading to the death of over 150 000
54 women worldwide ¹. Out of all ovarian cancers, high-grade serous ovarian, peritoneal or fallopian-tube cancer
55 (HGSC) is the most common subtype and the cause of 70-80% of all ovarian cancer-related deaths ². The 5-
56 year overall survival of HGSC has remained poor, approximating less than 40%, with DNA-damaging therapy
57 and cytoreductive surgery as the standard of care ^{2,3}. The development of new immunological therapies and
58 patient stratification has been hampered by the lack of a detailed understanding of the tumor-immune
59 microenvironment in HGSC.

60 Characteristically 20-22% of HGSC tumors are deficient in *BRCA1* or *BRCA2* genes, either via germline or
61 somatic mutations, or hypermethylation ⁴. *BRCA1/2* mutations disrupt the high-fidelity homologous

62 recombination (HR) DNA repair pathway, and consequently these cells are dependent on error-prone DNA
63 repair mechanisms and sensitive to both DNA damaging agents and Poly-ADP Ribose Polymerase inhibitors
64 ⁵. In addition to the *BRCA1/2* mutations, HR-deficiency can be caused by mutations or epigenetic modifications
65 in other genes of the HR-pathway ⁵.

66 Notably, preliminary evidence suggests that HR-deficient tumors have a distinct tumor-immune
67 microenvironment ^{6,7}. *BRCA1/2*mut tumors have been predicted to contain more neoantigens than tumors with
68 no alterations in genes of the HR pathway (HRwt)⁶, harbor an increased number of tumor infiltrating
69 lymphocytes ⁶, and have an elevated PD-L1 expression as compared to HR-proficient tumors ^{6 8}. Consistent
70 with these findings, we recently reported enhanced responses to combination of PARP inhibitor plus PD-1
71 inhibitor in patients with HR-deficient tumors⁹. However, a comprehensive view of the tumor
72 microenvironment (TME) and the interplay of tumor, immune and stromal cells of *BRCA1/2* mutated tumors
73 has not yet been described.

74 We performed highly multiplexed imaging utilizing 21 markers in 44 HGSCs and a deep phenotypic profiling
75 of 124,623 single cells to uncover the roles of the tumor- immune- and stromal cell subpopulations in the
76 HGSC TME. We herein report phenotypically and spatially divergent microenvironments in the *BRCA1/2*mut
77 tumors as compared to the HRwt tumors, with distinct cellular subpopulations and their spatial interactions
78 contributing to clinical outcomes in *BRCA1/2* mutated HGSC.

79

80 **Materials and Methods**

81 Cohort description The clinical cohort consisted of 27 patients with *BRCA1* mutated tumors (20 germline, five
82 somatic and one not determined), 4 patients with *BRCA2* mutated tumors (3 germline, 1 somatic) and 13
83 patients with tumors without any alterations in the HR-genes. HR-genes included *ATM*, *ATR*, *BRCA1*,
84 *BRCA2*, *BRIP1*, *CHEK2*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *NBN*, *PTEN*, and *U2AF1*;
85 they were assessed using OncoPanel Next-generation sequencing assay as previously described ⁶. Tumors
86 harboring mutations in the nucleotide excision repair and mismatch repair pathways were excluded. In

87 addition, immunohistochemistry for *BRCA1* was used to exclude *BRCA1* promoter hypermethylation in tumors
88 without mutations in the HR genes ⁶. The histology of all tumors was confirmed as HGSC by an experienced
89 pathologist. The tumor material consisted of 112 tumor 1.0 mm cores on a single tissue-microarray (TMA);
90 mostly triplicate cores per patient.

91 Highly multiplexed imaging, and image processing. The samples were stained sequentially with the validated
92 antibodies (**Supplementary Table 1**) and scanned with RareCyte CytFinder scanner following the tCycIF
93 protocol ¹⁰. Scanned image files were corrected using the BaSiC tool and stitched and registered using the
94 ASHLAR algorithm (<https://github.com/labsyspharm/ashlar>) to align image tiles and successive images of
95 tiles from all cycles to one another. We detected and cropped the cores from the stitched multi-channel image
96 with Image Processing Toolbox on MATLAB (version 2018b, The MathWorks, Inc., Natick, Massachusetts,
97 United States). This process produced multi-channel .tiff files that were further converted for Ilastik using the
98 Fiji plugin ilastik4ij-1.7.3. Probability maps for segmentation were produced using Ilastik, where all pixel
99 features were used for class discrimination. We trained Ilastik with 10 cores on 3-class manually labeled data
100 (inner nuclei, cell membrane, image background). We segmented the resulting probability maps (exported as
101 unsigned 16-bit tif images with axis order tzyxc=[1, 1, 1400, 1400, 32]) and the simple segmentation results
102 for the label 'nuclei'. The results were processed in Fiji to produce a final set of masks by expanding nuclei
103 masks and removing objects with area < 30 pixels. We quantified log mean fluorescence intensity for each
104 channel as well as default morphological features with histoCAT software. The mean number of cells per core
105 was 1107, median 1030 and range 29 to 3282. Subsequently, the mean number of cells per sample was 2818,
106 median 2524 and range 278 to 5933. The number of cells per patient and the number of TMA-cores per patient
107 is presented in **Supplementary Figure 1A**. We used three channels imaged with secondary-only antibodies
108 (Rabbit 488, Rat 555, Mouse 647) with a background threshold of 97th percentile for quality control to remove
109 unqualified cells. We removed lost cells based on radical changes in the DNA channel across cycles as
110 previously described in Cycif Analysis Suite (https://github.com/yunguan-wang/cycif_analysis_suite). ROIs
111 with artefacts (e.g. bubble, blobs, debris) were identified by visual inspection in the Open Microscopy
112 Environment Omero, and were removed from further analysis. XY-plots of the marker expressions per single
113 cell to were further visualized to confirm data quality.

114 Cell phenotyping and spatial analysis. We utilized our recently published tool CYTO¹¹ to cluster and iteratively
115 assign cell type labels. The quantified cell data were clustered in CYTO, followed by annotation of the clusters
116 based on their median marker expression profiles. The clusters were visualized with heatmaps and minimum
117 spanning trees of marker expressions per cluster in CYTO, and with UMAP using marker expression per cell.
118 The process consisted of five stages: 1) separation of tumor cells from immune and stromal cells, 2) separating
119 immune cells from stromal cells, 3) immune cell subtyping, 4) tumor cell metacluster annotation, and 5)
120 stromal cell metacluster annotation. Cells that did not fit the criteria for any cell types were re-clustered or
121 finally labeled as unknown. Markers used to annotate tumor cells, stromal cells and immune cell subtypes are
122 presented in **Supplementary Figure 1b**.

123 To confirm the annotations were correct, the identities of the cells were visually confirmed by overlapping the
124 cell class on the original images using Napari image-viewer. The samples were stained for CD3, CD4 and
125 CD8, and scored by a pathologist for the average count of CD3+, CD4+ and CD8+T-cells of three selected
126 CD3+T-cell rich tumor areas per patient ⁶, and these annotations were compared with our image-analysis based
127 annotations. Simpson's diversity index (SDI) was calculated using R-package ([https://cran.r-](https://cran.r-project.org/web/packages/vegan/vignettes/diversity-vegan.pdf)
128 [project.org/web/packages/vegan/vignettes/diversity-vegan.pdf](https://cran.r-project.org/web/packages/vegan/vignettes/diversity-vegan.pdf)).

129 Lineage trajectory analyses for immune cell subtypes and tumor metaclusters were performed using R package
130 STEMNET (<https://git.embl.de/velten/STEMNET>)¹². For immune cells, macrophages expressing only either
131 IBA1, CD163, or CD11c were not considered developmental endpoints. For tumor metaclusters, all
132 metaclusters were annotated as developmental endpoints. Markers in the immune cell lineage trajectory plot
133 included immune cell type markers (IBA1, CD163, CD11c, CD4, CD8a, CD3d, FOXP3, CD20) and functional
134 markers (Ki67, P21, PD1, PDL1, cCasp3 and pSTAT1). Markers in tumor metacluster lineage trajectory plot
135 included the functional markers (Ki67, P21, PDL1, cCasp3 and pSTAT1), as well as E-cadherin and vimentin.

136 The cell-cell spatial neighborhoods were assessed using a cutoff of 30 pixels from the centroid of each cell to
137 the centroid of neighboring cells using rangesearch function in MATLAB, excluding itself from the resulting
138 list. The mean proportion of each cell type neighboring each specific centering cell type was calculated per
139 core, and these values were normalized by dividing the value by the proportion of the specific cell type per

140 core, after which the log2 fold changes were calculated between *BRCA1/2*mut and HRwt tumors to compare
141 their spatial cell-cell neighborhood frequencies.

142 Statistics

143 Statistical analyses were performed in R version 3.6.1. Unpaired two-tailed Wilcoxon tests were used to
144 calculate two-sided p-values for pairwise comparisons. Effect sizes were calculated for the differences in cell
145 type proportions between the two mutational groups. False discovery rate was controlled by the Benjamini-
146 Hochberg procedure for functional marker expression differences, for correlation matrix between the
147 proportions of the cell types and for the spatial analysis of fractions of neighbors for each cell type. Those
148 values passing FDR <0.1 are discussed in the text. Spearman correlation coefficients and their p-values were
149 used in correlation analyses. An Iowess regression was used in situations where linear regression was
150 inappropriate due to violation of assumptions. P-values less than 0.05 were considered significant. Kaplan-
151 Meier graphs were plotted according to the method using the survival and survminer packages in R, and
152 patients at risk with no events at the end of follow-up were censored. P-values were calculated with the log-
153 rank test. For immune subtype proportions, the median value was used as a cut-off for high- and low proportion
154 groups. For functional marker expressions, the top 1/3 percentile was annotated as high and the rest as low.
155 Cox-proportional hazards were calculated for the first 800 days. The proportional hazards assumption for Cox-
156 proportional hazards were tested using cox.zph function in the survival package in R, and models with values
157 less than 0.05 were considered not suitable for analysis.

158 Results

159 **Multiplexed imaging reveals tumor microenvironment composition at single-cell resolution**

160 We performed highly multiplexed imaging of 21 markers on a TMA with 112 cores (**Supplementary Table**
161 **1**) and quantified the data at single-cell resolution from 44 HGSCs (**Fig. 1a**). We annotated and categorized
162 the single cells to tumor, immune and stroma, and subsequently to functional cell subpopulations. The tumor
163 microenvironment (TME) compositions, cellular diversity and functional states were associated to patient
164 clinical outcomes. The clinical characteristics of the patients are shown in **Table 1**. As expected, the platinum-

165 free interval (PFI), calculated as the time from last platinum-based chemotherapy to tumor relapse, was
166 significantly longer in patients with *BRCAl/2*mut tumors as compared to patients with HRwt tumors in this
167 patient cohort ($p=0.02$, log-rank test, HR 0.30 (95% CI 0.13-0.70), **Fig. S1c**). Overall survival (OS) was not
168 significantly different between the groups ($p=0.3$, log-rank test, HR 0.38 (95% CI 0.11-1.26), **Fig. S1d**).

169 The markers used for annotating the cell types in the TME are shown in **Fig. S1b**. Prior to clustering with
170 FlowSOM ($k=20$), the data were sample-wise normalized using mean-centering. First, tumor cell clusters were
171 separated, and the remaining cells were further clustered to detect and annotate clusters by their stromal and
172 immune marker expression. The immune-annotated cells were first divided into lymphocytes and myeloid
173 cells with FlowSOM ($k=16$). Lymphocytes yielded four subtypes (CD8+T-cells, CD4+T-cells,
174 CD4+FOXP3+T-regulatory cells and CD20+B-cells) with PhenoGraph ($knn=60$, markers CD8a, CD4,
175 FOXP3 and CD20). Myeloid cells we assigned into six subtypes (IBA1+CD163+, IBA1+CD163+CD11c+,
176 IBA1+CD11c+, IBA1+ and CD163+ macrophages, and CD11c+ antigen presenting cells) using FlowSOM
177 ($k=16$). Separately, tumor cells were clustered using k-means ($k=25$, markers E-cadherin, Ki67, P21, PDL1,
178 cCasp3, PSTAT1 and Vimentin) and annotated into seven metaclusters: epithelial cells, proliferating epithelial
179 cells, epithelial-mesenchymal-transition (EMT) cells, proliferating EMT cells, mesenchymal cells, apoptotic
180 cells and functional epithelial cells. Stromal cell metacluster annotation with k-means ($knn=25$, markers
181 vimentin, CD31, Ki67, P21, PDL1, cCasp3, pSTAT1 and cell eccentricity – defined as the ratio of the distance
182 between the focus points of an ellipse to its major axis length) produced nine metaclusters: high-proliferative
183 stroma, proliferative stroma, non-proliferative stroma, high-vimentin, low-vimentin, low-eccentricity,
184 functional stroma, high-p21 stroma and endothelia.

185 Out of all 124,623 cells, 70% were classified as tumor cells, 15% immune cells, 12% stromal cells and 3%
186 remained unannotated (Images, cell segmentation masks, and single-cell data can be found at DOI:
187 [10.7303/syn23747228](https://doi.org/10.7303/syn23747228)). As expected, each tumor was characterized by a unique TME composition, with tumor
188 cells being the dominant cell type in 40 out of 44 patients (**Fig. 1b**). UMAP shows that the tumor cells formed
189 patient-specific clusters, whereas the immune, stromal and endothelial cells formed more common clusters
190 between patients (**Fig. 1c and Fig. 1d**). To further characterize the tumor cells according to their functional
191 states, we classified the 85,621 tumor cells into seven metaclusters using minimum spanning trees (**Fig. S3a**).

192 The two most abundant metaclusters were proliferating epithelial (29.9%) and epithelial (29.6%) cells,
193 followed by EMT (15.3%) and proliferating EMT (11.4%) phenotypes (**Fig. 1e and Fig. 1f**), and each sample
194 contained cells belonging to a minimum of five different tumor metaclusters (**Fig. 1e**).

195 We next classified the 19,697 immune cells into distinct immune-cell subtypes. Of the immune cells,
196 IBA1+CD163+ macrophages were among the most abundant (29.3%), followed by CD8+T-cells (18.1%) (**Fig.**
197 **1g**). Each immune cell subtype showed consistent marker expression profiles (**Fig. S1e**) and visual clustering
198 (**Fig. 1h**). Further, the immune cells followed the canonical trajectories in lineage analysis (**Fig. S1f**).
199 Importantly, the proportions of CD3+ and CD8+T-cells in the imaging-based single-cell data correlated with
200 the scoring from conventional immunohistochemistry evaluated from fields selected for high CD3+T-cell
201 content, providing additional evidence on the accuracy of the automated imaging-based cell type classification
202 (**Fig. S1h-j**).

203 We next classified the 15,402 stromal cells into nine metaclusters using functional markers and morphological
204 features (**Fig. 1j, Fig. S1k**). In contrast to tumor- and immune compositions, the stromal cells showed a
205 heterogeneous composition without a dominant metacluster in any tumor (**Fig. 1i**). However, a subset of the
206 patients formed a cluster characterized by an enriched proportion of proliferative stromal cells (**Fig. S1g**).
207 Furthermore, the *BRCAl/2mut* tumors had a higher overall ratio of tumor cells to stromal cells as compared to
208 the HRwt tumors ($p = 0.038$, **Fig. S1l**).

209 Via iterative clustering approaches, we were able to separate the 124,623 single cells into phenotypic tumor
210 metaclusters, immune subtypes, and stromal metaclusters. Each tumor was characterized by a unique TME
211 composition, with tumor cells forming the most abundant cell population in almost every tumor. In contrast to
212 tumor metaclusters and immune subtypes, the stromal metaclusters showed no dominant metacluster in any
213 tumor.

214

215

216

217 **Distinct immune cell subtypes associate with immune diversity and clinical outcomes in HGSC HR-** 218 **genotypes**

219 Profiling of the immune cell subpopulations revealed highly variable immune compositions across the patients.
220 Using hierarchical clustering of the proportions of different immune cell subtypes out of all immune cells, we
221 observed that a cluster of tumors harboring an IBA1+CD163+ macrophage –dominant immune composition
222 was almost exclusively *BRCA1* mutated ($p=0.045$, Fisher’s exact test, **Fig. 2a**).

223 Consistently, the *BRCA1/2*mut tumors exhibited a significantly higher infiltration of IBA1+, CD163+ and
224 IBA1+CD163+ macrophages as compared to the HRwt tumors (Wilcoxon $p=0.027$, $r=0.34$, **Fig. 2b, Fig. S2a**).
225 Additionally, we noted a trend of increased infiltration of CD8+T-cells in the *BRCA1/2*mut tumors when
226 compared to the HRwt (Wilcoxon $p=0.056$, $r=0.29$, **Fig. S2b**). Deeper analysis of the immune compartment
227 of the tumors revealed distinct myeloid cell profiles with the HRwt tumors exhibiting increased proportions of
228 CD11c+ antigen presenting cells (CD11c+ APC, Wilcoxon $p=0.0013$, $r=0.47$), and IBA1+CD163+CD11c+
229 macrophages (Wilcoxon $p=0.00067$, $r=0.49$) out of all immune cells as compared to *BRCA1/2*mut tumors (**Fig.**
230 **2c and Fig. 2d**). These findings led us to explore the ratios of CD8+ T-cells to macrophages and antigen-
231 presenting cells in the TME of the different HR-genotypes. Consistently, we observed an increased ratio of
232 CD8+T-cells to both IBA1+CD163+CD11c+ macrophages (Wilcoxon $p=0.0043$, $r=0.46$, **Fig. 2e**) and to
233 IBA1+CD11c+ macrophages (Wilcoxon $p=0.0012$, $r=0.43$, **Fig. S2c**) in the *BRCA1/2*mut tumors as compared
234 to the HRwt tumors, indicative of enriched T-cell mediated immunosurveillance over CD11c+ myeloid cell
235 populations in the *BRCA1/2*mut tumors as compared to the HRwt tumors.

236

237 We next examined whether the proportions of the different immune cells associate with PFI or OS using a
238 median cut-off for high or low proportions of the immune cell infiltration out of all cells. Consistent with
239 previous findings^{13,14}, higher CD8+T-cell infiltration was associated with longer PFI in all patients (**Fig. 2g**,
240 $p=0.015$, log-rank test, HR 0.27, 95% CI 0.11-0.63), but not with OS. However, this difference was not
241 significant when the analysis was limited to only patients with *BRCA1/2*mut or HRwt tumors (**Fig. S2d and**
242 **S2e**). Interestingly, a higher infiltration of CD4+T-cells was associated with longer PFI, also separately in

243 patients with *BRCA1/2*mut tumors (**Fig. 2h**, $p=0.0011$, log-rank test, HR 0.26, 95% CI 0.10-0.66, **Fig. S2g**).
 244 This finding was not significant in patients with HRwt tumors (**Fig. S2f**). Additionally, a high infiltration of
 245 FOXP3+CD4+T-regulatory cells was associated with an improved PFI in all patients as well as in patients
 246 with *BRCA1/2*mut tumors, and a high infiltration of IBA1+CD163+ macrophages in all patients (**Fig. S2h-**
 247 **2m**). However, in multivariate Cox regression models in patients with *BRCA1/2*mut tumors and in all patients
 248 pooled using the significant cell types from Kaplan-Meier analysis as variables, only CD4+T-cells were
 249 significant and independently associated with PFI (*BRCA1/2*mut: $p=0.043$, HR 0.24 (95% CI 0.06-0.95), All
 250 patients: $p=0.02$, HR 0.34 (95% CI 0.14-0.85), **Supplementary Table 2**).

251
 252 We next calculated a measure of intratumoral diversity for immune cell subtypes using Simpson's diversity
 253 index (SDI). Overall, the SDI for immune cells was higher in the HRwt tumors as compared to the
 254 *BRCA1/2*mut tumors (Wilcoxon $p=0.0053$, $r=0.41$, **Fig. 2f**), and the SDI did not correlate with the total immune
 255 cell infiltration (**Fig. S2p**). In the *BRCA1/2*mut tumors, the SDI for immune cells correlated positively with
 256 the proportions of IBA1+CD163+CD11c+ (Spearman $p=0.019$, $R=0.42$, **Fig. 2j**) and IBA1+CD11c+
 257 macrophages and CD11c+ APCs (Spearman $p=0.00002$, $R=0.70$, **Fig. S2n** and Spearman $p=0.00001$, $R=0.71$,
 258 **S2o**), suggesting that a more diverse immune microenvironment associates with increased presence of cells
 259 with antigen presentation capacity. By contrast, SDI for immune cells correlated negatively with
 260 IBA1+CD163+ macrophage proportions out of immune cells in both of the tumor HR-genotypes
 261 (*BRCA1/2*mut Spearman $p=0.0014$, $R=-0.56$, HRwt: $p=0.027$, $R=-0.62$ **Fig. 2i**). Immune diversity was
 262 however not associated with PFI in the two clinical groups (**Fig. S2q-s**).

263
 264 Further investigation of the immune suppressive signals in the TME revealed an overall higher expression of
 265 PD-L1 in the macrophage and antigen presenting cell subtypes as compared to stromal (Wilcoxon FDR
 266 corrected $p<2.2\times10^{-16}$) and tumor cells (Wilcoxon FDR corrected $p<2.2\times10^{-16}$) (**Fig. S2t**). Importantly, the PD-
 267 L1 expression was significantly higher in the CD11c+ APCs (Wilcoxon FDR corrected $p=2.6\times10^{-9}$) and
 268 IBA+CD11c+ macrophages (Wilcoxon FDR corrected $p<2.2\times10^{-16}$) in the *BRCA1/2*mut, and in

269 IBA1+CD163+CD11c+ macrophages (Wilcoxon FDR corrected $p=2.4 \times 10^{-5}$) in the HRwt tumors.
270 Furthermore, the expression of PD1 was significantly higher in CD8+T-cells (Wilcoxon FDR corrected
271 $p=0.00044$), CD4+T-cells (Wilcoxon FDR corrected $p=0.00044$), and FOXP3+CD4+T-regulatory cells
272 (Wilcoxon FRD corrected $p<2 \times 10^{-16}$) in *BRCAl/2*mut as compared to HRwt tumors (**Fig. S2u**). Functional
273 marker expressions for the immune cell subtypes are presented in **Supplementary Figure 2v**.

274

275 Altogether, the analysis of the immune microenvironment revealed marked differences in cell type
276 compositions between the HR genotypes, with distinct myeloid cell compartments, associations to immune
277 diversity and PD-1/PD-L1 expression profiles. The proportions of CD8+T-cells associated with improved PFI
278 in all patients pooled, and CD4+T-cells also separately in patients with *BRCAl/2*mut tumors.

279

280 **Functional states of tumor cell metaclusters associate with distinct outcomes in the HR-genotypes**

281 We next examined the tumor single-cell phenotypes using tumor cell metaclusters annotated by their functional
282 marker expression. In hierarchical clustering of the proportions of tumor cell metaclusters out of all tumor
283 cells, epithelial and proliferating epithelial metaclusters clustered together whereas the EMT and proliferating
284 EMT formed a unique cluster, suggesting that either epithelial or EMT state is favored within each tumor (**Fig.**
285 **3a**). By visual inspection of a minimum spanning tree constructed from the original cell clusters, proliferating
286 epithelial cell clusters and proliferating EMT cell clusters were separated from epithelial and EMT cell clusters
287 into separate branches (**Fig. 3b**), underscoring the differential proliferative states between these groups (**Fig.S**
288 **3a**). The functional marker expression of each metacluster is presented in **Figure 3d**.

289 In Lineage trajectory analysis of the tumor cell functional states, the epithelial and EMT endpoints were
290 projected on the opposite simplex corners (**Fig. 3c**). The epithelial cells were projected between functional and
291 proliferating epithelial cells, while the EMT cells were projected between the mesenchymal and proliferating
292 EMT cells reflecting the continuous nature of the EMT process. The mesenchymal tumor cells showed a
293 lineage towards the EMT cells as well as directly towards proliferating EMT cells. The apoptotic cells were

294 projected between the proliferating epithelial and proliferating EMT cells. The lineage trajectory plots colored
295 by Ki67 and vimentin are presented in Supplementary Figure 3b and c.

296 Interestingly, the *BRCA1/2*mut tumors contained overall an increased proportion of proliferating epithelial
297 cells out of all cells as compared to the HRwt tumors (Wilcoxon $p=0.0053$, $r=0.38$, **Fig. 3e**). Moreover, the
298 HRwt tumors contained an increased proportion of EMT cells out of all cells as compared to the *BRCA1/2*mut
299 tumors (Wilcoxon $p=0.024$, $r=0.37$, **Fig. S3d**), however the difference in the total EMT proportion out of all
300 cells was not significant when taking into account also proliferating EMT cells (**Fig. S3e**). A subset of patients
301 displayed a dominantly mesenchymal or epithelial phenotype as their most abundant tumor metacluster, and
302 further the cluster characterized by high epithelial cell content associated with low tumor cell metacluster SDI
303 (Wilcoxon $p=0.013$, $r=0.43$, **Fig. 3a**). The tumor cell metacluster proportions did not associate with clinical
304 outcomes (data not shown). The tumor SDI was similar between the HR genotypes, and did not correlate with
305 the proportion of tumor cells out of all cells (data not shown), or PFI (**Fig. S3f-h**).

306

307 Interestingly, the functional states of the tumor cells were differentially associated with PFI in the HR-
308 genotypes (**Fig. 3f-g**). Notably, the expression of the proliferation marker Ki67 affected PFI in an opposite
309 direction in the *BRCA1/2*mut tumors as compared to the HRwt tumors (**Fig. 3f and 3g**). In Kaplan Meier
310 analysis stratified by the highest 1/3rd of median Ki67 expression in the proliferating epithelial cells, a high
311 expression of Ki67 was associated with a prolonged PFI (**Fig. 3h**, $p = 0.029$, log-rank, HR 0.12, 95% CI 0.02-
312 0.88) in patients with *BRCA1/2*mut tumors. By contrast, we observed an opposite trend in patients with HRwt
313 tumors, where a low expression of Ki67 in proliferating epithelial cells was associated with a better PFI (**Fig.**
314 **S3j**, $p = 0.013$, log-rank, HR 0.10 95% CI 0.10-0.90).

315

316 Altogether, the tumor cells were characterized by an abundance of proliferating epithelial cells in the
317 *BRCA1/2*mut tumors, and EMT cells in the HRwt tumors. Hierarchical clustering of the tumor metacluster
318 proportions suggested that either epithelial or EMT lineage is favoured within each tumor. Lineage trajectory
319 analysis showed a continuum of the EMT process, and apoptotic cells stemming from proliferating epithelial

320 and proliferating EMT cells. Importantly, we found an opposing prognostic role for Ki67 expression in
321 proliferating epithelial cells in the HR genotypes, with a higher median Ki67 associating with an improved
322 PFI in *BRCA1/2*mut tumors.

323 324 **Cell subpopulations distinctively co-occur in the TME of the HR genotypes**

325 In hierarchical clustering of all cell type proportions (**Fig 4a**), the cell types clustered into two main groups,
326 the first one characterized by tumor EMT and mesenchymal metaclusters, IBA1+ macrophages, and stromal
327 metaclusters, and the second one characterized by other tumor metaclusters and immune cell subtypes,
328 suggestive of a different patterns of immune activity in the tumor-rich and stromal-rich TMEs. Importantly,
329 clustering analysis revealed differences in the overall cellular composition in the *BRCA1/2*mut and HRwt
330 tumors (**Fig 4a**). HRwt tumors were enriched in a cluster characterized by enhanced stromal presence from
331 high-proliferative, high-P21, endothelial, non-proliferative, high-vimentin, functional, low-vimentin, low-
332 eccentricity and proliferative stromal cells ($p=0.003$, Fisher's exact test). Interestingly, as subset of the
333 *BRCA1/2*mut tumors were characterized by high infiltration of CD8+ and CD4+ T-cells and B-cells.

334 To further investigate the interactions of the tumor metaclusters, immune subtypes and stromal metaclusters
335 in the HR-genotypes, we plotted the correlations between the proportions of cell types separately for
336 *BRCA1/2*mut and the HRwt tumors (**Fig. 4b**). Overall, in the *BRCA1/2*mut tumors, the tumor metaclusters had
337 more significant correlations with immune subtypes ($n=6$) than with the stromal cell metaclusters ($n=0$). By
338 contrast, in the HRwt tumors, the stromal cell metaclusters had as many significant correlations with immune
339 subtypes ($n=4$) as with the tumor cell metaclusters ($n=4$), suggesting distinct shaping of the immune
340 microenvironment by the tumor cell subpopulations in *BRCA1/2*mut tumors.

341
342 Overall, the proportions of CD8+T-cells and CD4+T-cells were highly correlated with each other in both of
343 the HR-genotypes (*BRCA1/2*mut: Wilcoxon FRD corrected $p=2.1 \times 10^{-5}$, HRwt: $p=0.069$), and had similar
344 correlations with other cell types (**Fig. 4b**). In the *BRCA1/2*mut tumors, CD8+T-cells correlated positively
345 with IBA1+CD11c+ macrophages (Wilcoxon FDR corrected $p=0.0004$), CD4+ T-cells correlated positively
346 with CD11c+ APCs (Wilcoxon FDR corrected $p=0.00022$), and both CD8+ T-cells and CD4+ T-cells

347 correlated positively with B-cells (Wilcoxon FRD corrected $p=2.5 \times 10^{-9}$, $p=6.8 \times 10^{-5}$) suggestive of a TME with
348 coordinated immune activity. In the HRwt tumors, high-proliferative stroma correlated negatively with
349 CD4+T-cells (Wilcoxon FRD corrected $p=0.07$), and the CD4+T-cells correlated positively with
350 IBA1+CD163+ macrophages (Wilcoxon FRD corrected $p=0.047$), suggesting that the stromal compartment
351 may contribute to enhanced immunosuppression in the HRwt tumors.

352

353 To further explore the relationships between the cell types, we computed Principal Component Analysis
354 (PCA)-plots separately for the *BRCA1/2*mut and the HRwt tumors (**Fig. 4c and 4d**). In both the *BRCA1/2*mut
355 and the HRwt tumors, the first principal components were mainly characterized by either high immune
356 infiltration or high stromal infiltration. In the *BRCA1/2*mut tumors, the second principal component was mainly
357 characterized by proliferative epithelial and mesenchymal tumor metaclusters, with proliferating epithelial
358 cells opposing the mesenchymal metacluster cells and the immune subtypes. In the HRwt tumors, the second
359 principal component was characterized by stromal metaclusters and the mesenchymal tumor metacluster,
360 which oppose the proinflammatory IBA1+CD11c+ macrophages. The similar direction of the loading vectors
361 of immune cells in the PCA-plots, especially in the *BRCA1/2*mut tumors, suggested a coordinated immune
362 system with simultaneous recruitment of multiple immune cell subtypes. Consistently, the PCA biplot
363 indicated that patients with a long PFI generally harbored tumors with rich immune infiltration, whereas
364 patients with a short PFI were characterized by tumors rich in stromal metaclusters (**Fig. 4e and 4f**). The third
365 principal component revealed similar direction of EMT, proliferating EMT and high-proliferative stromal cell
366 metaclusters opposing the epithelial cell metacluster in the *BRCA1/2*mut tumors (**Fig. S4a and b**). In HRwt
367 tumors, the third principal component showed opposing direction of the epithelial cell metacluster and CD11c+
368 APCs, FOXP3+CD4+ T-regulatory cells as well as functional and low-eccentricity stroma (**Fig. S4c and d**),
369 further supporting the stromal contribution on immunosuppression in the HRwt tumors.

370 The PCA-plots with all patients pooled further supported the distinct TMEs in the two HR-genotypes; the first
371 two principal components display the HRwt tumors characterized by stromal metaclusters and the
372 *BRCA1/2*mut tumors characterized by immune cell subtypes and proliferating epithelial cells (**Fig. S4e and f**).

373 The third principal component, characterized by EMT and proliferating EMT metaclusters opposing CD163+
374 macrophages and endothelial cells, showed further separation of the HR genotypes (**Fig. S4g and h**).

375

376 Altogether, the combined analysis of all cell types showed differential TME composition and cell type co-
377 occurrence profiles in the HR genotypes. CD8+ and CD4+T-cells correlated positively with other
378 immunostimulatory cells suggestive of coordinated recruitment of immune cells in the *BRCA1/2*mut tumors.
379 By contrast, the HRwt tumors were characterized by an increased presence of stromal metaclusters, their
380 negative association to CD4+T-cells and role in opposing immune cell infiltration in the PCA-plots.

381

382 **Spatial cell-cell interactions suggest enhanced immunosurveillance in *BRCA1/2* mutated tumors**

383 To uncover the physical interactions between the cell types, we explored the TME spatial organization by
384 calculating the fraction of neighboring cell types for each single cell and computed the mean of these fractions
385 per tumor to compare the cellular communities between the *BRCA1/2*mut and HRwt tumors. The neighborhood
386 fractions were normalized by their cellular abundancies in the corresponding tumor cores. Interestingly, we
387 observed marked differences in the spatial organization of the TME cell types in the *BRCA1/2*mut as compared
388 to the HRwt tumors. The correlation matrix and summary graphics are shown in **Fig. S5a**, and **Fig. 5a**. We
389 evidenced enhanced spatial interaction of the proliferating epithelial cells with CD8+T (Wilcoxon FDR
390 corrected $p=0.036$), CD4+T-cells (Wilcoxon FRD corrected $p=0.053$) and FOXP3+CD4+ T-reg (Wilcoxon
391 FRD corrected $p=0.070$) in the *BRCA1/2*mut tumors as compared to HRwt tumors. Also endothelial cells
392 (Wilcoxon FRD corrected $p=0.024$) and functional stromal cells (Wilcoxon FRD corrected 0.036) showed
393 enhanced spatial interactions with the proliferating epithelial cells in the *BRCA1/2*mut tumors (**Fig. 5a**). Visual
394 inspection of representative images revealed CD8+T-cells residing in the neighborhoods of proliferating
395 epithelial cells in a *BRCA1/2*mut tumor, whereas the TME was more compartmentalized with less cellular
396 interactions between the proliferating epithelial cells in a HRwt tumor (**Fig. 5b**).

397 In the *BRCA1/2*mut tumors, the CD4⁺T-cells resided predominantly in cellular communities rich in
398 proliferating epithelial cells (Wilcoxon FRD corrected $p=0.065$) and other CD4⁺T-cells (Wilcoxon FRD
399 corrected $p=0.049$). Further supporting the spatial immune-stimulatory TME of the *BRCA1/2*mut tumors, the
400 immune-suppressive FOXP3⁺CD4⁺ T-regs showed enhanced spatial interactions with non-proliferative
401 stromal cells (Wilcoxon FRD corrected $p=0.070$, $p=0.099$).

402 We found no significant differences in immune cells neighboring endothelial cells between the two HR
403 genotypes. However, endothelial cells were in spatial proximity with IBA1⁺CD163⁺ macrophages
404 significantly more often as compared to other immune cell subpopulations (**Fig. S5c**).

405

406 Additionally, we observed distinct spatial interactions between the myeloid cells and other cells of the TME.
407 In the *BRCA1/2*mut tumors, IBA1⁺CD163⁺ macrophage communities were enriched in proliferating epithelial
408 cells and epithelial cells (Wilcoxon FRD corrected $p=0.036$, $p=0.056$). By contrast, the CD163⁺ macrophages
409 neighbored functional stromal compartments in the HRwt tumors (Wilcoxon FRD corrected $p=0.0007$),
410 suggesting that these macrophages reside in functional stromal niches more often in HRwt than in *BRCA1/2*mut
411 tumors. Hierarchical clustering visualization of the normalized neighborhood fractions of the significant
412 bidirectional interactions further highlights the differential spatial TMEs in the HR genotypes (**Fig. S5b**).
413 Altogether, the distinct spatial cellular communities suggest enhanced immunosurveillance of proliferating
414 epithelial tumor cells by the CD4⁺ and CD8⁺T-cells in the *BRCA1/2*mut tumors as well as enhanced spatial
415 interactions of M2-macrophages with epithelial cells, and immune-suppressive M2-macrophage-stromal
416 interactions in the HRwt tumors.

417 Finally, we investigated the potential prognostic roles of the the spatial interactions of CD4⁺T-cells and
418 CD8⁺T-cells with proliferating epithelial cells. We found that both the fractions of CD4⁺T-cells and CD8⁺T-
419 cells in the neighborhood of proliferating epithelial cells were significantly associated with prolonged PFI in
420 patients with *BRCA1/2*mut tumors (**Fig. 5c and d**). In a multivariate cox regression analysis using the CD4⁺
421 and CD8⁺T-cell neighbor fractions of proliferating epithelial cells as well as the Ki67 status of proliferating
422 epithelial cells, none of these were, however, independently significantly associated with PFI or OS

423 (Supplementary Table 3). This suggests that, in addition to the Ki67 expression in epithelial tumor cells, the
424 coordinated immune surveillance in the spatial cellular communities contributes to better prognosis in the
425 *BRCA1/2*mut tumors.

426

427 **Distinct functional states of proliferating epithelial tumor cells associate with immune cell spatial** 428 **interactions in *BRCA1/2* mutated HGSCs**

429 The prognostic roles of CD8+ and CD4+T-cells, and their observed enhanced spatial interactions with
430 proliferating epithelial cells in the *BRCA1/2*mut tumors prompted us to investigate whether Ki67 expression
431 in proliferating epithelial tumor cells is associated with enhanced cell-to-cell spatial interactions. We compared
432 the Ki67 expression in the proliferating epithelial tumor cells neighbored by CD8+ or CD4+T-cells as
433 compared to cells without these cells as neighbors (Fig. 5e). Interestingly, we observed a higher Ki67
434 expression in the proliferating epithelial cells with one or more CD8+T-cells as neighbor as compared to cells
435 without any CD8+T-cells as neighbors in the *BRCA1/2*mut tumors (Wilcoxon $p=0.0026$, $r=0.02$), but not in
436 the HRwt tumors (Fig. 5f, Fig. S5d). A similar phenomenon was observed in proliferating epithelial tumor
437 cells with CD4+T-cells as neighbors in patients with *BRCA1/2*mut (Wilcoxon $p=1.1 \times 10^{-5}$, $r=0.03$) but not with
438 HRwt tumors (Fig. 5g, Fig. S5e). Collectively, these findings suggest that in the *BRCA1/2*mut tumors, high
439 Ki67 expression in proliferating epithelial cells results in improved immunosurveillance, with enhanced cell
440 to cell interaction with CD4+T-cells and CD8+T-cells, whereas in the HRwt tumors the high-proliferative
441 tumor cells are not spatially interacting with CD4+ or CD8+T-cells. These distinct spatial tumor-immune
442 interactions provide potential insights into the mechanisms driving the differential prognostic impact of Ki67
443 expression in proliferative epithelial cells in tumors with different HR-genotypes.

444

445 **Discussion**

446 Lack of detailed information on the interplay of tumor, stromal and immune cells in the tumor
447 microenvironment in HGSC has been lacking. Herein, we aimed to shed more light into how *BRCA1/2*

448 mutations and single-cell tumor phenotypes shape the immune microenvironment and prognosis in HGSC.
449 Using imaging-based single-cell data, we captured and annotated 124,623 cells of the tumor, stromal and
450 immune cell populations in the TME of 44 HGSC patients. We discovered distinct immune microenvironments
451 and divergent interactions between the immune subtypes and tumor and stromal metaclusters in tumors with
452 *BRCA1/2*mut as compared to HRwt tumors. Interestingly, we found opposing prognostic roles of proliferative
453 functional states of epithelial tumor cells in patients with *BRCA1/2*mut and HRwt tumors, which also
454 associated with distinct cellular neighborhoods. The graphical summary of the distinct TME phenotypes in the
455 *BRCA1/2*mut and the HRwt tumors is presented in **Figure 6**.

456

457 Single-cell immunophenotyping revealed divergent immune cell compartments in the HR genotypes.
458 Consistent with previous observations from syngeneic mouse models⁷, the *BRCA1/2*mut tumors contained an
459 increased infiltration of IBA1+ and M2-type IBA1+CD163+ and CD163+ macrophages, whereas the HRwt
460 tumors had abundant CD11c+ myeloid cells. The myeloid cells also showed abundant and distinct PD-L1
461 expression profiles between the HR genotypes, with the highest expression in the CD11c+ cells in the
462 *BRCA1/2*mut tumors and M2-type macrophages in the HRwt tumors. Overall, the infiltration of CD8+ and
463 CD4+T-cells correlated positively with the infiltration of various macrophages, as suggested also previously¹⁵.
464 Further, consistent with previous reports^{6,7,16}, we observed a trend towards increased CD8+T-cell infiltration
465 in the *BRCA1/2*mut tumors, and a balance favoring CD8+T-cell infiltration over infiltration of multiple
466 myeloid cell types in the in the TME of *BRCA1/2*mut tumors, suggesting enhanced immunosurveillance
467 capacity via CD8+T-cells. Importantly, the CD8+T-cells were characterized by a higher PD-1 expression in
468 the *BRCA1/2*mut tumors implicating potentially enhanced susceptibility to PD-1/PD-L1 targeted immune
469 checkpoint therapies. By contrast, the lower CD8+T-cell infiltration patterns strengthen the notion that T-cell
470 exclusion is more common in the HRwt tumors.

471

472 Investigation of the prognostic roles of the immunophenotypes revealed that a higher infiltration of both
473 CD8+T-cells and CD4+T-cells were associated with prolonged responses to platinum-based chemotherapy,

474 consistent with previous findings^{13,14}. Importantly, in multivariable analysis the CD4+T-cell infiltration
475 displayed an independent prognostic role, also in patients with *BRCA1/2*mut tumors. Interestingly, the CD4+T-
476 cells had more immune stimulatory correlations with CD11c+ myeloid cells and B-cells in the *BRCA1/2*mut
477 tumors. In addition, the proportions of CD11c+ antigen presenting cells correlated with increased diversity of
478 other immune cells, and the co-occurrence of tumor infiltrating T-cells, B-cells, and antigen presenting cells
479 suggested a more coordinated immune cell activity in the *BRCA1/2*mut tumors. Further, spatial analysis
480 revealed enhanced self-interactions of the CD4+T-cells in the *BRCA1/2*mut tumors as compared to the HRwt
481 tumors. Altogether, these findings suggest a coordinated, immune-stimulatory role for CD4+T-cells in the
482 *BRCA1/2* mutated HGSCs.

483

484 Lack of single-cell resolution on large-enough clinical cohorts has hampered the in-depth investigation on the
485 role of tumor cell subpopulations and their functional states on patient prognosis. Previous studies have
486 reported increased proliferative states associated with *BRCA1* mutation in breast^{17,18}, and ovarian tumors¹⁹.
487 However the reports on the potential prognostic role of high Ki67 expression in HGSC have remained
488 conflicting²⁰⁻²². In the present study, the proliferating epithelial cells formed the most abundant tumor cell
489 subpopulation, which was further enriched in the *BRCA1/2*mut tumors. Interestingly, a high Ki67 expression
490 in proliferating epithelial cells conveyed an opposing prognostic effect in the HR-genotypes; high expression
491 of Ki67 in the proliferating epithelial cells associated with a longer PFI in patients with *BRCA1/2*mut tumors,
492 but with a trend for shorter PFI in the HRwt tumors. This difference can be, at least in part, explained by the
493 differential platinum sensitivity due to HR-deficiency: the highly proliferating epithelial cells are likely to be
494 more sensitive to platinum-based chemotherapy in the *BRCA1/2*mut tumors as compared to the HRwt tumors.
495 Thus, using single-cell resolution information in tumors stratified by the predicted HR capacity, we were able
496 to resolve the previously conflicting conclusions, and revealed potentially clinically significant role of tumor
497 cell functional proliferative state with the premise to promote the development of prognostic and predictive
498 biomarkers for HGSC.

499 Our unique single-cell dataset enabled further characterization of the associations of the cellular functional
500 states and their spatial interactions. Analysis of the cellular neighborhood frequencies revealed a higher
501 proliferative state of the epithelial cells residing in CD4⁺ or CD8⁺T-cell cellular communities in the
502 *BRCAl/2*mut tumors. By contrast, the epithelial cells with a higher proliferative state were residing outside of
503 the CD4⁺ or CD8⁺T-cell neighborhoods in the HRwt tumors. The underlying mechanism behind the enhanced
504 spatial immunosurveillance is likely to involve increased interferon activation²³, and increased neoantigens⁶ in
505 the *BRCAl/2*mut tumors. In line with previous reports^{15,24}, the proportions of proliferating epithelial tumor
506 cells correlated negatively with CD8⁺ and CD4⁺T-cell infiltration, indicating that the immune surveillance of
507 CD8⁺ and CD4⁺T-cells towards the highly proliferating epithelial cells in the *BRCAl/2*mut tumors is regulated
508 by spatial attraction and not just by their cellular abundance.

509 Consistently, the enhanced cellular communities of both CD4⁺T-cells and CD8⁺T-cells with the proliferating
510 epithelial cells associated with a better PFI in patients with *BRCAl/2*mut tumors. Further, the prognostic role
511 of the CD8⁺T-cells was conveyed only by their spatial arrangement, and not by relative abundance in
512 *BRCAl/2*mut tumors. Importantly, the multivariate survival analyses confirmed that the prognostic role of the
513 Ki67 expression in the proliferating epithelial cells is not independent from the spatial CD8⁺ and CD4⁺T-cell
514 communities, implicating that coordinated and spatial interplay of the cell subpopulations is needed to convey
515 prognostic relevance. Our single-cell spatial analyses of the TME thus illuminate the mechanisms on how both
516 the tumor cell genotypes and immune infiltration contribute to prognosis in HGSC²⁵.

517

518 The role of the stromal cells in HGSC genotypes has also remained elusive^{15,26}. We observed that the HRwt
519 tumors contained an increased stromal to tumor ratio, and significant intercorrelations of the stromal
520 metaclusters with the immune cell subpopulations, suggesting enhanced stromal contribution to the
521 immunophenotypes and T-cell exclusion. Stromal cells promote the presence of physical and biochemical
522 barriers which can further contribute to therapy resistance^{15,27,28}. Moreover, we found that the HRwt tumors
523 contained an increased proportion of tumor cells with an EMT phenotype, which also could contribute to

chemoresistance²⁹⁻³¹. Altogether, the stromal dominance, tumor cell EMT functional states, and decreased immune surveillance can contribute to the poor prognosis of the HRwt tumors.

We acknowledge certain limitations of the study. Our patient cohort consisted of a limited number of HRwt tumors as compared to the *BRCA1/2*mut tumors, rendering the conclusions made on the *BRCA1/2*mut tumors stronger. Further, most of the *BRCA1/2*mut tumors were *BRCA1* mutated (n=27). Although the TME analysis using a tissue microarray minimizes experimental variation, it poses a potential bias on the conclusions regarding tumor heterogeneity as only a maximum of three 1mm cores can be examined, and thus we had to refrain from performing permutation-based spatial analyses which require a larger dataset size, and focus on the communities based on normalized neighbor fractions for each cellular community. Further, the experimental design requires the application of a limited set of protein-based markers, thus leaving a small proportion of the cells (average 5% per tumor) unannotated. In addition, the pioneering nature of the work has hampered availability of similar cohorts or datasets, and thus validation in an external cohort, preferably using an unbiased method such as spatial single-cell RNAsequencing, would be needed to strengthen the conclusions of the study.

In conclusion, single-cell analysis highlights the functional and spatial differences in the TME landscapes of *BRCA1/2*-deficient and HRwt tumors, underscoring the need for differential treatment strategies for these clinical subgroups. Uncovering the effects of tumor genotypes on the TME, cellular phenotypes and spatial communities will enable the development of more effective immunotherapeutic strategies and improved patient stratification in HGSC.

548 **Author contributions**

549 I-M.L analyzed the data and wrote the manuscript; N.L, J.C., E.A.A and A.S analyzed the data and wrote the
550 manuscript; C.A.J. and J.R.L performed the experiments and wrote the manuscript; U-M.H, Z.M; S.S; K.A;
551 A.D.A; P.A.K and P.K.S provided materials and resources, supported the data analysis and wrote the
552 manuscript; A.F conceived and supervised the study and wrote the manuscript.

553

554 **Competing interests statement**

555 The authors report no competing interests.

556

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Figure 1

Fig 1. Multiplexed imaging reveals tumor microenvironment composition at single-cell resolution

a A schematic representation of the workflow, from tumor tissue microarray (TMA) composition and high-dimensional imaging to cell type calling, population description, spatial and functional analysis, and clinical correlations. Using a TMA, a total of 112 cores from 31 patients with *BRCA1/2*mut tumors and 13 patients with tumors without any mutations in HR genes were imaged with 21 markers. After image processing, labels from cell type calling were used for description of immune subtypes as well as tumor and stromal metaclusters. These profiles were in turn used in diversity analysis. The spatial and functional states of cell types were analyzed and correlations with clinical information were evaluated. **b** Annotated barplot of the tumor, immune, stromal, and endothelial cell composition per patient, showing high tumor content across patients. The barplot is sorted by decreasing tumor proportion. Clinical annotations for barplots include HR status and PFI, with a PFI of <12 months annotated as short and > 12 months long. **c** UMAP visualizing tumor, immune, stromal, and endothelial cells as distinct populations with color annotations corresponding to Fig. 1b, and **d** colored by their patient of origin. The most abundant subtype were tumor cells, followed by stromal, immune, and endothelial cells. **e** Annotated barplot describing the tumor metacluster composition across different patients and clinical groups, showing heterogeneity across patients. The barplot is sorted by decreasing proliferating epithelial cell proportion. **f** UMAP visualizing the tumor cell metaclusters as distinct populations. **g** Annotated barplot describing the immune subtype composition across different patients and clinical groups, with a heterogeneous immune composition between patients. The barplot is sorted by decreasing IBA1+CD163+ macrophage proportion. **h** UMAP visualizing the immune cells as distinct populations, with cells of lymphoid and myeloid lineages grouping to the opposite sides of the UMAP. **i** Annotated barplot describing the stromal metacluster composition across different patients and clinical groups, showing less heterogeneity across patients than tumor metaclusters and immune cell subtypes. The barplot is sorted by decreasing non-proliferative stroma content. **j** UMAP visualizing the stromal cell metaclusters as distinct populations.

Figure 2

a

b

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Fig. 2 Distinct immune microenvironments associate with immune diversity and clinical outcomes in HGSC

a A hierarchical clustering heatmap of immune subtype proportions out of all immune cells reveals distinct immune-microenvironments, annotated with immune diversity (immune SDI), and clinical data including HR status and PFI. The barplot annotations for the columns and rows represent the total number of cells per each immune cell subtype and the number of the immune cells in total per patient, respectively. **b** Boxplot showing the combined proportion of IBA1+, CD163+ and IBA1+CD163+ macrophages as a proportion of all cells, stratified by HR status. **c** Boxplot showing the increased proportion of CD11c+ antigen-presenting cells as well as **d** increased proportion of IBA1+CD163+CD11c+ macrophages as a proportion of all immune cells in HRwt as compared to *BRCAl/2*mut tumors **e** Boxplot showing increased ratio of CD8+T-cells to IBA1+CD163+CD11c+ macrophages in *BRCAl/2*mut as compared to HRwt tumors. **f** Boxplot showing higher immune diversity in HRwt as compared to *BRCAl/2*mut tumors. The differences between the boxplot groups were calculated by Wilcoxon signed-rank test. The black horizontal lines represent the sample medians, the boxes extend from first to third quartile and whiskers indicate the values at 1.5 times the interquartile range. Individual dots represent values per each tumor. **g** Kaplan-Meier graphs for PFI show improved PFI for high CD8+T-cell proportion in all tumors pooled as well as **h** for high CD4+T-cell proportion in patients with *BRCAl/2*mut tumors. Proportions were calculated out of all cells, and the median value was used to distinguish high and low groups. Number of patients at risk is shown at the bottom of each Kaplan-Meier graph. The comparisons between the groups were performed using the log-rank test. **i** Scatter plots with lowess regression show negative correlation of immune diversity and the proportion of IBA1+CD163+ macrophages in all HR groups and **j** a positive correlation of immune diversity and the proportion of IBA1+CD163+CD11c+ macrophages in *BRCAl/2*mut tumors. Proportions were calculated out of immune cells and Spearman correlation coefficients and their p-values are shown. HR groups include *BRCAl/2*mut (blue), HRwt (red) tumors and all tumors pooled (black).

Figure 3

Fig. 3 Tumor metacluster functional states associate with clinical outcomes in *BRCA1/2*mut tumors

a A hierarchical clustering heatmap of tumor metacluster proportions out of all tumor cells shows distinct tumor microenvironments, annotated with tumor SDI representing tumor diversity, and clinical data including HR status and PFI. The barplot annotations for the columns and rows represent the total number of cells per each tumor metacluster and the number of tumor cells in total per patient, respectively. **b** Minimum-spanning tree of the original tumor cell clusters with circles visualizing the assigned metaclusters based on median marker expression profiles. Smaller metaclusters are marked with letters. Annotations include the assigned metaclusters. **c** Lineage trajectory plot taking all tumor cell metaclusters as endpoints shows both separation and interconnections between epithelial and EMT phenotypes. **d** Heatmap of tumor metaclusters showing distinct expression levels (Z-score) of the markers in the metaclusters. Rows of the heatmap are sorted by metacluster abundance. **e** Boxplot showing the increased proportion of proliferating epithelial cells as a proportion of all cells in *BRCA1/2*mut as compared to HRwt tumors. The differences between the groups were calculated by Wilcoxon signed-rank test. Boxplots visualize the sample medians, first to third quartile and the values at 1.5 times the interquartile range. Individual dots represent values per each tumor. **f** Dot plot of fold changes ($\log_2$) of functional marker expression in tumor metaclusters in *BRCA1/2*mut tumors, between long and short PFI and **g** in HRwt tumors between long and short PFI. The color of the dots represents the fold change and their size the significance of the p-value. Fold changes with FDR corrected p-values <0.05 are shown. Blue boxes highlight the differential Ki67 expression in proliferating epithelial cells in long and short PFI tumors, in the different HR genotypes. **h** Kaplan-Meier graphs showing high Ki67 expression in proliferating epithelial cells associates with an improved PFI in patients with *BRCA1/2*mut tumors. Patients belonging to the top 1/3 percentile in all tumors in median Ki67 expression were annotated as high and the rest as low. Number of patients at risk is shown at the bottom of each Kaplan-Meier graph. The comparisons between the groups were performed using the log-rank test.

Figure 4

Fig. 4 HR-genotypes show different patterns of cell type co-occurrence with CD8+T-cells and CD4+T-cells

a A hierarchical clustering heatmap of all cell proportions shows enrichment of HRwt tumors in the cluster characterized by stromal metaclusters. Annotations include the HR status, PFI group, as well as the global cell type of the metaclusters. **b** A Spearman correlation dot plot between tumor metaclusters, immune subtypes and stromal metaclusters as proportions out of all cells, divided by HR status. Dots in white background and light-red background represent correlations in *BRCAl/2*mut and HRwt tumors, respectively. Dots shaded red represent positive correlations while dots shaded blue represent negative correlations. Black lines separate the tumor, immune and stromal cell columns and rows. Blue dashed boxes highlight the interactions that CD4+ T-cells and CD8+ T-cells have with other cell types. Correlations with a p-value <0.05 are shown and those passing FDR 0.1 are highlighted with black squares. **c** Principal component feature projections for *BRCAl/2*mut tumors and **d** for HRwt tumors. 12 cell types with the highest contributions to PC1 and PC2 are shown for each plot. The more intense the color of the cell type, the coefficient is. **e** Plot of patients with *BRCAl/2*mut tumors and **f** plot of patients with HRwt tumors projected onto their first two principal components. Patients are annotated as having a long or short PFI and colored by light blue or dark blue, respectively. Ellipsoids show the 95% confidence intervals for the PFI groups.

a

Fig. 5 Proliferative state delineates spatial cell-cell interactions of the tumor-immune microenvironment in *BRCA1/2*mut and HRwt tumors

a A schematic representation of cell-cell spatial interactions significantly enriched ($FDR < 0.1$) in *BRCA1/2*mut tumors (blue arrows) and in HRwt tumors (red arrows). The arrow heads point to the cell type surrounding the cell type denoted by the arrow base. The size of the arrows indicate the FDR-corrected p-value. The dotplot in Fig. S5a denotes the corresponding fold change values. **b** Representative multiplex immunofluorescence images showing highly proliferating epithelial cells co-localizing with CD8+T-cells in *BRCA1/2*mut tumors, and highly proliferating epithelial cells residing further away from CD8+T-cells in HRwt tumors. **c** Kaplan-Meier graphs showing high fraction of CD4+T-cells and **d** high fraction of CD8+T-cells neighboring proliferating epithelial cells associate with an improved PFI in patients with *BRCA1/2*mut tumors. Median was used as a cut-off for high and low fractions. Number of patients at risk is shown at the bottom of each Kaplan-Meier graph. The comparisons between the groups were performed using the log-rank test. **e** A schematic illustration of functional state and spatial cell-cell interaction analysis. Proliferating epithelial cells were each characterized by their CD4+T-cell and CD8+T-cell spatial neighborhood status. Next, Ki67 marker expression was compared in proliferating epithelial cells with different cell-cell spatial neighborhoods in *BRCA1/2*mut and HRwt tumors separately. **f** Violin plots showing the probability densities of Ki67 expression in proliferating epithelial cells in the different CD8+T-cell neighborhood and HR-groups. **g** Violin plots showing the probability densities of Ki67 expression in proliferating epithelial cells in the different CD4+T-cell neighborhood and HR-groups. In the box plots inside violin plots the black horizontal lines represent the sample medians, the boxes extend from first to third quartile and whiskers indicate values at 1.5 times the interquartile range. Wilcoxon signed-rank test was used for pairwise comparisons. P-values: $<0.05 = *$, $<0.005 = **$, $<0.0005 = ***$, $<0.00005 = ****$.

Figure 6

Fig. 6 Graphical summary of the differences in the TME of *BRCA1/2*mut and HRwt tumors

A schematic representation of the differences in cell type infiltration and their spatial interactions in the *BRCA1/2*mut and HRwt tumors, characterized by proliferating epithelial and EMT tumor cells, macrophages and antigen presenting cells, as well as the spatial interactions of CD8+T-cells and CD4+T-cells with differently Ki67 expressing proliferating epithelial cells and other immune cells. Curved arrows represent a spatial interaction.

Tables

Table 1. Summary of patient clinical characteristics

Variable	Category	mean	median	range
Age at diagnosis (years)	All patients	58.1	59.1	42.2-80.0
	<i>BRCAl/2</i> mutated	55.2	53.7	42.2-75.6
	HRwt	65	64.3	49.1-80.0
PFI (months)	All patients	39.4	14.3	0.7-271.7
	<i>BRCAl/2</i> mutated	49.5	24	6.2-271.7
	HRwt	15.3	6.9	0.7-110.4
OS (months)	All patients	66.4	44.6	5.8-276.7
	<i>BRCAl/2</i> mutated	82.1	53.3	7.5-276.7
	HRwt	29	17.9	5.8-116.3

Figures

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Tumor metacluster functional states associate with clinical outcomes in BRCA1/2mut tumors a A hierarchical clustering heatmap of tumor metacluster proportions out of all tumor cells shows distinct tumor microenvironments, annotated with tumor SDI representing tumor diversity, and clinical data including HR status and PFI. The barplot annotations for the columns and rows represent the total number of cells per each tumor metacluster and the number of tumor cells in total per patient,

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Supplementary Files

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