Polyclonal and clonal organoid models of Barrett oesophagus and oesophageal adenocarcinoma reveal heterogeneity in progression and therapy response

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HIGHLIGHTS

1. Patient- and healthy donor-derived organoids (PDOs) provide a functional platform of disease progression and heterogeneity across normal gastric, non-dysplastic and dysplastic Barrett oesophagus (BO) and oesophageal adenocarcinoma (OAC).
2. We provide a quantitative phenotypic and molecular framework to assess the provenance and fidelity of each PDO model given the heterogeneity of this disease.
3. PDOs recapitulate key features of non-dysplastic and dysplastic BO, as well as invasive OAC.
4. Single cell-derived clonal organoids (sc-organoids) isolate and maintain high-risk subclonal populations.
5. OAC PDOs mirror known population level variation in response to systemic anti-cancer therapies.

SUMMARY/ABSTRACT

Oesophageal adenocarcinoma (OAC) is a major cause of morbidity and mortality. OAC and its precursor, Barrett oesophagus (BO), are defined by substantial early heterogeneity, complicating prevention and treatment of OAC and remaining poorly recapitulated by current *in vitro* and animal model systems. We have generated 116 patient- and healthy donor- derived organoids (PDOs) spanning normal oesophagogastric tissue, BO and OAC. These PDOs capture population diversity and recapitulate phenotypic, genomic and transcriptomic features of their respective disease stages. We develop a single cell-derived clonal organoid approach and show that this enables us to capture the heterogeneity and isolate high-risk, subclonal populations that are difficult to discern and maintain in bulk PDO cultures. Using this platform, we demonstrate functional importance of this biobank across the pre-malignant to invasive disease spectrum, including a role for BO in shaping fibroblast phenotype within assembloids, and diverse responses of OAC to chemotherapy, radiotherapy and targeted CDK4/6 inhibition.

Key words: Barrett oesophagus; Dysplasia; Oesophageal adenocarcinoma; Organoid; Assembloid; Heterogeneity; Systemic anti-cancer therapy; Chemotherapy; Radiotherapy

INTRODUCTION

Oesophageal adenocarcinoma (OAC) is an important cause of cancer-related morbidity and mortality, with an overall five-year survival rate of less than 20%[1]. Around 85,000 people are diagnosed with OAC each year, corresponding to a global age-standardised incidence of 0.9 cases per 100,000 persons[2]. Following increases in incidence since the 1970s, OAC is now the dominant subtype in over 20 high income countries, where rates reach as high as 3.4 cases per 100,000 people[2, 3].

The development of OAC follows the replacement of the normal stratified oesophageal squamous epithelium with a metaplastic mosaic of gastric and intestinal like epithelium[4]. This lesion, Barrett oesophagus (BO), originates from cells within the gastric cardia, likely in response to exposure to injurious gastric refluxate[4, 5]. BO typified by intestinal metaplastic features can progress to dysplasia and invasive disease but this occurs in a minority of patients, with an annual risk of OAC of just 0.3% for a person with a non-dysplastic lesion[6]. Greater phenotypic and molecular variation also correlates with the risk of progression to OAC[7-9]. A more precise understanding of events leading to progression is vital to enabling early detection and interception strategies[10]. The heterogenous nature of the BO precursor lesion gives rise to a highly diverse cancer at phenotypic and molecular levels of resolution[11-13]. This tumour heterogeneity establishes a therapeutic landscape with few recurrent actionable drivers for targeted therapies and high levels of resistance to conventional treatments and immunotherapy, leading to poor outcomes[10, 14].

There is consequently a need to develop an experimental platform that recapitulates inter- and intra-lesional heterogeneity from the development of BO through to its dysplastic progression to OAC. In this context, animal models are poorly representative and do not reproduce between-patient heterogeneity[15]. The use of animal models is also costly and resource intensive, with multiple ethical implications that have prompted calls to move away from their routine use. By contrast, we have previously shown that OAC patient-derived organoids (PDOs) can be established[16]; however, the extent to which these also reproducibly demonstrate heterogeneity in therapeutic response to established therapies including cytotoxic chemotherapy and external beam radiotherapy (EBRT) is less certain[17, 18]. PDOs also lack stromal factors that are known to contribute to disease pathogenesis and treatment response, such as the presence of fibroblasts[19, 20]. Moreover, establishing these models from precancerous lesions has proven more challenging[16]. The aim of this study was therefore to establish and characterise a PDO biobank of

sufficient scale to recapitulate the full spectrum of heterogeneity at each stage of disease progression, including normal gastric epithelium, non-dysplastic and dysplastic BO, and treatment-naïve as well as treated OAC. Using a combination of genomic sequencing and *in silico* modelling of PDO growth and morphology, we provide a quantitative toolkit to characterise each organoid. To address intralesional heterogeneity in BO, we also introduce novel, single cell-derived clonal organoids (sc-organoids) to unmask minor cell populations at high risk of dysplastic progression that may be missed in bulk analyses or lost through successive passages. We show that co-culture with fibroblasts (termed assembloids) and microenvironmental stressors results in altered epithelial-fibroblast interactions and the reprogramming of stromal components. Finally, we demonstrate that OAC PDO phenotypic and molecular heterogeneity translates to variation between PDOs in their response to different forms of cytotoxic chemotherapy, EBRT and targeted therapy.

RESULTS

Establishment of PDOs spanning BO and healthy comparison tissues

The first aim was to develop a robust and representative PDO resource spanning the entire spectrum of BO development and progression prior to OAC development. Thirteen PDOs were derived from non-dysplastic BO segments at routine surveillance endoscopy and eight PDOs were derived from punch biopsies from regions of dysplasia in endoscopic mucosal resection specimens **(Supplementary Table 1)**. Respective efficiencies for deriving PDOs were therefore 68% (13/18) and 61% (8/13) from non-dysplastic and dysplastic tissue. In addition,16 normal gastric PDOs were established, including seven from deceased healthy organ gastroesophageal squamocolumnar junction (n=4) and gastric body (n=3) donor tissues **(Supplementary Table 2)**. Nine further gastric PDOs were derived from patients with BO: one each from the gastric body and antrum, and seven from the cardia to give a range of gastric lineages. Three further PDOs were derived from normal intestinal tissue as control for the intestinal phenotype, which characterises BO with malignant potential. All PDOs were passaged at least five times, and all were successfully revived following cryopreservation.

BO PDOs recapitulate population heterogeneity and key features of non-dysplastic as well as dysplastic disease

We first sought to understand the genomic heterogeneity of the BO PDOs in relation to dysplasia status and in comparison to matching tissue samples. Median somatic mutation burden was comparable and reflected disease stage, with 2.11 muts/Mb in non-dysplastic PDOs versus 1.89

69 muts/Mb in matching tissues, and 5.76 muts/Mb in dysplastic PDOs versus 4.57 muts/Mb in
70 matching tissues. Median burden in normal stomach was 0.11muts/Mb and 0.26 muts/Mb from
71 healthy- and patient- derived PDOs respectively **(Supplementary Figure 2, A and B)**. As
72 expected, PDOs showed higher purity, defined as the proportion of epithelial, precancerous
73 cells in a sample, and higher clonality than their matching tissues, which was achieved in non-
74 dysplastic and dysplastic models **(Supplementary Figure 2, C and D)**.
75
76 A comprehensive overview of OAC driver genes, mutational signatures, and clinical annotations
77 is provided in **Figure 1A**. As expected, there were few mutational events in normal gastric PDOs.
78 In contrast, *CDKN2A* alterations, primarily deletions, were identified in 31% of non-dysplastic
79 and 38% of dysplastic PDOs, consistent with the reported role of this gene in early BO
80 development[43]. Alterations in *TP53* were seen in only one (1/13, 8%) non-dysplastic BO PDO but
81 were more frequent (n=3/8; 37.5%) in PDOs taken from patients with dysplasia.
82
83 The fraction of shared mutations between BO PDOs and their matching tissues ranged from 0.3-
84 65.3% **(Figure 1B)**. Broadly, the mean fraction of private mutations (i.e. that are not shared) in
85 PDOs was higher in dysplastic PDOs than in non-dysplastic PDOs (median 56.9% vs 38.1%).
86 Whilst there was no correlation between the total number of insertion/deletion mutations in
87 PDOs and matching tissue (r=0.034, p=0.89), there was a moderate correlation in single
88 nucleotide variant (SNV) count (r=0.66, p=0.0015; **Figure 1C**) which was stronger for non-
89 dysplastic PDO/tissue pairs (r=0%.91, $p=3.4x10^{-5}$) **(Supplementary Figure 2E)**. These results
90 suggest increasing heterogeneity in dysplastic compared with non-dysplastic tissues which is
91 thus reflected in the variation captured within a given PDO model.
92
93 Most tissues and PDOs were diploid; however, we were keen to capture the whole genome
94 doubling observed in up to 27% high-grade dysplastic BO tissues[44]. Three PDOs had whole
95 genome doubling. This was mirrored in matching tissue for one PDO (AHM0931) but not in the
96 other two (AHM2575 (non-dysplastic) and AHM2165 (dysplastic), which featured whole genome
97 duplications despite diploid matching tissues **(Figure 1D)**. Overall concordance in homologous
98 recombination deficiency (HRD) index scores was low (r=0.15, p=0.53); however, again non-
99 dysplastic pairs showed a moderate positive correlation (r=0.52, p=0.081), though dysplastic
100 pairs did not (r=–0.34, p=0.41). This suggests that it is challenging to capture chromosomal
101 instability in PDOs and one cannot rely on the genomic profile of matching tissues
102 **(Supplementary Figure 2F)**.

In comparison to the accumulation of driver gene mutations, mutational signatures are laid down early in the pathogenesis of BO and maintained consistently through malignant progression[45]. It was therefore expected to observe a high concordance of mutational signatures in the PDOs and matching tissues, with a median cosine similarity of 0.97 (range 0.73-1.00), which did not differ between dysplastic and non-dysplastic PDO-tissue pairs **(Figure 1E and Supplementary Figure 2, G and H).** In particular, single base substitution signature 17 (SBS17a/b), a characteristic feature of BO and OAC, showed a strong overall correlation for both SBS17a ($r=0.83$, $p=6.2x10^{-6}$) and SBS17b ($r=0.88$, $p=3.8x10^{-7}$) **(Figure 1F, Supplementary Figure 2I)**. As expected, SBS17a/b proportions were similar across non-dysplastic and dysplastic PDOs **(Supplementary Figure 2J)**.

We also observed clonal heterogeneity and drift in precancerous PDOs **(Supplementary Figure 2, K and L).** For example, AHM0040 contained a major subclonal population (VAF 18%) harbouring *ARID1A* and *FBXW7* driver mutations. In AHM1373, a subclonal cluster (median VAF ~19%) was present at passage 1 but became clonal by passage 6 (median VAF 50%). Whilst no driver alterations were present in both passages, passage 1 harboured a mutation in *LRKK2* **(Figure 1A**), whilst passage 6 carried frameshift variants in *ARID1A*, *ARID1B*, and *MUC6*, possibly indicating selection of a separate, rarer subclone. Hence, these models evolve temporally, and this can be tracked genetically as required for their utility to study disease progression and interception.

Single cell-derived BO PDOs recapitulate intralesional heterogeneity

In view of the substantial genomic heterogeneity and the passage-related clonal drift we identified in BO PDOs, we reasoned that isolating low-frequency, high-risk populations would enable single clone modelling of progression. To achieve this, we developed a microdissection method to derive individual organoids from bulk PDO culture. We termed these single cell-derived clonal organoids (sc-organoids) **(Figure 2A).**

We first generated 19 sc-organoids from a non-dysplastic case (AHM2149) and confirmed their clonality by analysing VAF distributions (a narrow peak at 50% indicates clonality) **(Figure 2B)**. The sc-organoid panel recapitulated key genomic features of matching BO tissue as well as the related bulk PDOs, including deletions of *CDKN2A* and *KDM6A,* and mutations in *MUC6* and *ZFHX3* **(Figure 2C)**. Interestingly, one sc-organoid, BE.11, harboured mutations in *TP53* and *SMARCA4* that were not observed in the bulk PDO nor its matching tissue, suggesting a potential

137 “seed” for dysplastic progression. All sc-organoids contain private mutations (3.44%-45.8%),
138 confirming that each represents a distinct lineage with preserved mutational signatures
139 **(Supplementary Figure 3, A and B)**.
140
141 We next assessed the relatedness of the sc-organoids by constructing a phylogenetic tree using
142 WGS data **(Figure 2D)**. Most mutations (85.98%) fitted the tree perfectly with minimal homoplasy,
143 and driver alterations showed good concordance within the branching structure, reflecting their
144 individual acquisition along the evolutionary trajectory. For example, *FBXW7* loss was shared by
145 BE.7 and BE.15 but absent in the bulk PDO, whereas a *ZFHX3* mutation was detected in the bulk
146 PDO but found in only four closely related sc-organoids.
147
148 To determine whether closely related sc-organoids also display phenotypic similarity, we
149 compared transcriptomic concordance with WGS-inferred phylogenies (**Figure 2E**). We also
150 assessed whether sc-organoids represent distinct lineages by analysing enrichment of individual
151 lineage markers (*MUC6* versus *CDX2*) and lineage gene panels (described in **Figure 3A**). Across
152 19 non-dysplastic BO sc-organoids, *MUC6* (gastric) and *CDX2* (intestinal) expression were
153 strongly negatively correlated ($r = -0.71$; $p = 0.00075$, **Supplementary Figure 3C**), indicating that
154 individual sc-organoids tend to adopt either a gastric or intestinal state. Notably, two sc-
155 organoids (BO.8 and BO.23) displayed diverging transcriptional profiles to the rest of the sc-
156 organoids, with significantly higher *CDX2* expression.
157
158 With these encouraging data we generated a panel of nine sc-organoids from dysplasia tissue
159 from an early cancer case (AHM2392), from which a bulk PDO shared only 8.15% of SNVs and
160 indels **(Figure 1B),** to better understand and model the clonal heterogeneity. All sc-organoids
161 appeared clonal, with the exception of one (BE.14) that also harboured a nonsense mutation in
162 *APC* present at a VAF of 25% **(Supplementary Figure 4A),** suggesting it may be a mixture of two
163 clones. Taken together these sc-organoids shared a large proportion of mutations with the
164 matching bulk PDO **(Supplementary Figure 4B)**, whilst also presenting with more mutations than
165 the bulk PDO on account of their high clonality **(Supplementary Figure 4C)**. The individual sc-
166 organoids nevertheless demonstrated clonal diversity **(Supplementary Figure 4D)** and
167 transcriptomic heterogeneity **(Supplementary Figure 4E)**, which with the isolation of an *APC*-
168 mutant clone reaffirms the potential for this technique to isolate high-risk populations not
169 captured in bulk PDOs **(Supplementary Figure 4F)**.
170

171 ***In silico* imaging reveals varying morphology, growth kinetics and between non-dysplastic**
172 **and dysplastic BO PDOs**
173 The genomic profile must be interpreted in the context of PDO morphology and growth patterns
174 to understand how this translates to phenotype. To assess morphology, we genetically
175 engineered non-dysplastic (AHM0040) and dysplastic (AHM2221) PDOs with nuclear fluorescent
176 tags (H2B-GFP/mCherry) and imaged using time-lapse microscopy. Unlike the characteristic
177 solid morphology of OAC PDOs[16], non-dysplastic PDOs showed four characteristic
178 morphologies: a single cell layer surrounding a single lumen, a single cell layer with multiple
179 lumens, multiple cell layers with a lumen and a solid morphology with no visible lumen **(Figure**
180 **3, A to C)**. Luminal PDOs expanded over time, whereas solid structures showed minimal growth
181 **(Supplementary Table 3).** In contrast, dysplastic PDOs lack single cell layer multiple lumen
182 morphology and have a higher proportion of solid morphology compared with non-dysplastic
183 PDOs **(Figure 3D, Supplementary Figure 5, A to C; Supplementary Table 4)**.
184
185 We observed minimal growth in almost all solid non-dysplastic organoids but visible expansion
186 in luminal types, measured by both organoid size and nuclear number **(Supplementary Figure 5,**
187 **D and E)**. Moreover, multiple and single lumen non-dysplastic PDOs demonstrated a similar,
188 linear growth rate with considerable variation in individual PDO growth **(Supplementary Figure**
189 **5, F and G)**. Growth kinetics of single-lumen PDOs were comparable between non-dysplastic and
190 dysplastic models **(Figure E, Supplementary Figure 5H)**. Time-lapse imaging also suggested
191 that multi-lumen PDOs might be a result of fusion events from single luminal PDOs, providing a
192 novel insight into the changing clonality of bulk organoid culture over time **(Supplementary**
193 **Figure 5, I and J).**
194
195 Lumen formation has been linked to polarity, the loss of which is associated with dysplastic
196 progression[46]. We established a lattice-based multi-site Cellular Potts Monte Carlo *in silico*
197 model of organoid growth in which organoid polarity, and the probability of a daughter organoid
198 exhibiting polarity, were varied **(Supplementary Figure 6A)**. Using this model, we demonstrate
199 that a single layer single lumen morphology is acquired where organoids and their progeny
200 demonstrate polarity, whereas organoids without polarity develop solid non-luminal structures
201 **(Supplementary Figure 6B)**. Variation in the probability of acquiring polarity results in a variability
202 in lumen formation **(Figure 3F)**. The model also accurately recreated the effects of observed
203 fusion processes **(Supplementary Figure 6, C to E)** and captured the loss of polarity in dysplastic
204 and OAC PDOs **(Supplementary Table 5).**

205

206 **Barrett oesophagus PDOs offer a functional platform for premalignant studies**

207 To extend the phenotypic characterisation we used transcriptomic profiling based on lineage
208 markers determined from our previous scRNA-seq datasets[5, 47]. As expected, the majority of BO
209 PDOs demonstrated an intermediate phenotype between that seen for gastric and intestinal
210 PDOs **(Figure 4A, Supplementary Figure 7, A and B)**. When comparing the bulk transcriptomes
211 of normal and precancerous PDOs using a principal component analysis (PCA), BO and intestinal
212 PDOs show a clear separation from gastric PDOs, in keeping with the acquisition of an intestinal
213 phenotype **(Figure 4B)**. When comparing differentially expressed genes (DEGs) between three
214 paired comparisons: gastric versus non-dysplastic PDOs, gastric versus dysplastic PDOs, and
215 non-dysplastic versus dysplastic PDOs, broad concordance was observed in non-dysplastic and
216 dysplastic PDOs when compared with gastric, while a small number of genes distinguished
217 dysplastic and non-dysplastic PDOs **(Figure 4, C and D, Supplementary Figure 7, C to E)**.
218 Pathway analyses revealed that upregulated genes in both non-dysplastic and dysplastic PDOs
219 were enriched for developmental, migratory, proliferative, and intestinal differentiation programs
220 **(Figure 4E, Supplementary Table 6)**, whereas down-regulated genes mapped to cell-peripheral
221 processes and transcription factor binding sites **(Figure 4E, Supplementary Table 6)**. In addition,
222 upregulation of extracellular matrix–related genes were specifically enriched in non-dysplastic
223 PDOs **(Supplementary Figure 7F, Supplementary Table 7),** while in dysplastic PDOs, down-
224 regulated genes mirrored the transcription factor binding site enrichment observed in the shared
225 down-regulated profiles **(Supplementary Figure 7G, Supplementary Table 8)**. Intriguingly, three
226 *HOX* genes (*HOXB2, HOXB6, HOXB8*), showed increased expression in dysplastic versus non-
227 dysplastic PDOs **(Figure 4, D and F)**, with a similar pattern also observed for *HOXB5* and *HOXB7*
228 **(Supplementary Figure 7H)**. This is consistent with our prior work demonstrating a role for HOX
229 genes in OAC carcinogenesis[48].

230

231 **Assembloid epithelial-fibroblast co-cultures recapitulate the role of fibroblasts in BO**
232 **development**

233 We have previously shown that BO acquires a pro-tumourigenic, activated fibroblast
234 environment, characterised by a reduction in the $CD34^+$ S1 fibroblast subset and an increase in
235 the αSMA^+ S3 subset[47]. However, the origin of this shift in fibroblast composition remains
236 unclear[19]. We reasoned that naïve fibroblasts could be reprogrammed into a BO-favoured niche
237 through a combination of intrinsic and extrinsic factors. To explore this, we built on a previously
238 described model to embed human primary oesophageal fibroblasts with BO or control normal

239 gastric PDOs **(Supplementary Figure 8A)**[49]. The resultant assembloids closely resembled
240 primary tissue, including with respect to glandular structures and stromal deposition
241 **(Supplementary Figure 8B)**.
242
243 We next sought to determine whether BO PDOs, either alone or in combination with pathological
244 microenvironmental stressors, could induce fibroblast reprogramming. To test this, we
245 characterised fibroblast subtypes in assembloids subjected to treatments mimicking reflux and
246 chronic local inflammation, which are the two key environmental factors associated with the
247 development and progression of BO. For experimental consistency, we used chemically defined
248 glycocholic acid, a bile acid component known to be highly effective in promoting BO
249 development[50]. In addition, we re-analysed our previous RNA-seq data to identify a relevant pro-
250 inflammatory exposure and selected interleukin 10 (IL10), a cytokine that shows a significant and
251 progressive increase during BO and OAC progression **(Supplementary Figure 9)**[51].
252
253 After treatment with reflux salt and/or IL10 for 7 days PDOs were dissociated into single cells and
254 analysed by flow cytometry **(Supplementary Figure 8C).** Reflux acid and IL10 treatment generally
255 reduced the proportion of S1 fibroblasts and increased the proportion of S3 fibroblasts
256 **(Supplementary Figure 8, D and E)**. However, this effect was much stronger in assembloids with
257 BO PDOs, which showed a markedly greater capacity to polarise fibroblasts towards the S3
258 subtype, particularly under reflux acid exposure. In contrast, normal gastric PDOs required both
259 reflux acid and IL10 to induce S3 fibroblasts, and the effect was comparatively modest.
260 **(Supplementary Figure 8F)**.
261
262 **OAC PDOs recapitulate population-level molecular heterogeneity with variable growth**
263 **behaviours**
264 The phenotypic and molecular heterogeneity that defines Barrett oesophagus is also a
265 characteristic feature of OAC[13]. Expanding on our previous small panel of 10 OAC PDOs[16], we
266 show here that a biobank of 38 PDOs (donor characteristics outlined in **Supplementary Table**
267 **11**) recapitulates the genomic landscape and known driver gene landscape for OAC[11, 52] **(Figure**
268 **5, A and B, Supplementary Figures 10 and 11)**. Mirroring prior studies from patient samples[11, 52],
269 alterations in PDOs were enriched for copy-number alterations (22.2%) rather than point
270 mutations or small insertion-deletion mutations, even using stringent thresholds for
271 amplifications and deletions. As with patient series, *TP53* was the most frequently altered gene
272 (70%) in the profiled PDOs, followed by *CDKN2A* (22%), *ARID1A* (18%), *GATA6* (18%), and *SMAD4*

273 (10%), although again concordance with matching tissues varied and 16% (n=4/25) showed
274 absence of a *TP53* mutation, compared with *TP53*-mutant matching tissues **(Figure 5C and**
275 **Supplementary Figure 11)**.
276
277 As in BO, there was broad alignment in the mutational signature landscape of PDOs and matching
278 tissues (median cosine similarity 0.98, range 0.74-1.00; **Figure 5D**). All previously reported[53] OAC
279 signatures (SBS17a/b, SBS18, SBS40, SBS1 and SBS5) were present **(Supplementary Figure 10I),**
280 with strong PDO–tissue concordance in the BO and OAC-defining[53, 54] SBS17a ($r=0.92$, $p=4.1\times10^{-}$
281 ^{14}) and SBS17b ($r=0.92$, $p=5.7\times10^{-14}$) signatures **(Supplementary Figure 10J)**. Compared with their
282 premalignant equivalents, OAC PDOs displayed increased SBS17a/b, SBS40, and marginal
283 increases in APOBEC-related SBS2/13 **(Supplementary Figure 10K)**. The OAC PDO
284 transcriptomic profile was variable, though there we did not observe specific gene expression
285 signatures that distinguished different groups of PDOs **(Figure 5E, Supplementary Figure 10, L**
286 **and M).** We also observed recurrent, ploidy-adjusted copy number gains (≥2 relative to sample
287 ploidy) in key oncogenes, including of the receptor tyrosine kinase HER2 in three PDOs, with
288 related protein overexpression **(Supplementary Figure 12).**
289
290 To compare OAC PDOs with BO growth metrics, we again used high-resolution time-lapse
291 microscopy **(Supplementary Figure 13, A to C)** Unlike BO, organoids with a solid structure
292 represented 99% of OAC PDOs observed at all assessed timepoints **(Supplementary Table 9)**.
293 Single cells contributing to OAC PDOs were significantly larger than those forming BO PDOs
294 **(Supplementary Figure 13D)** but demonstrated a similar growth rate **(Supplementary Figure**
295 **13E)**. A majority of PDOs grew in the seven days after seeding and did so with minimal underlying
296 variation in their growth rates **(Supplementary Figure 13, F and G)**, while some organoids more
297 rapidly increased in size because of fusion events **(Supplementary Figure 13H)**.
298
299 **OAC PDOs reproducibly show variable responses to anti-cancer therapies in keeping with**
300 **their molecular and phenotypic heterogeneity**
301 For OAC a key question is whether PDO phenotypic and molecular diversity translates to
302 differences between PDOs in their sensitivity to specific forms of systemic anti-cancer therapy
303 as well as external beam radiotherapy (EBRT), and whether the individual characteristics of each
304 PDO result in differences in sensitivity to individual chemotherapeutics, targeted agents and
305 EBRT. To assess this, five PDOs derived from treatment-naïve donors (OESO117, OESO146,
306 CAM277, CAM408, CAM574) were first exposed to the microtubule inhibitor (docetaxel), anti-

307 metabolite (5-fluorouracil (5-FU), with leucovorin) and platinum (oxaliplatin) constituents of the
308 current perioperative standard-of-care regimen, FLOT **(Figure 6A, Supplementary Table 10)**.
309 Mean IC_{50} values were lowest for docetaxel and demonstrated minimal variation between studied
310 PDOs (1.64–3.04 nM), whereas IC_{50} values for 5-FU/leucovorin (8.31–51.33 μM) and oxaliplatin
311 (IC_{50} 7.73–96.78 μM) were higher and demonstrate substantial variation. In contrast,
312 5FU/leucovorin E_{max} values were 93% or above in all but two PDOs but varied between 62.89-
313 95.82% for oxaliplatin, and between 50.04-93.53% for docetaxel. Validating the utility of using
314 PDOs as a pharmacokinetic model, there was no correlation between IC_{50} and E_{max} values for 5-
315 FU/leucovorin (r=0.10, p=0.87), oxaliplatin (r=-0.83, p=0.085) or docetaxel (r=0.51, p=0.38). We
316 also demonstrated a heterogeneity of response to external beam x-ray irradiation **(Figure 6B,**
317 **Supplementary Table 12)**. Organoid forming efficiency varied between 0.42-1.03 in response to
318 a standard 2Gy single fraction of EBRT, and between 0.24-0.75 in response to a high 8Gy single
319 fraction. This corresponded to an integral area under the curve (AUC) for response to 0-8Gy
320 single-fraction irradiation of between 0.46-0.98.
321
322 We and others have also previously reported that as many as 50% of cases of OAC contain
323 sensitising events for CDK inhibitors[11]. However, the extent to which this translates to a
324 consistent therapeutic response across an otherwise heterogenous population is uncertain.
325 Using four PDOs with relevant vulnerabilities (CAM277, CAM401, CAM429, CAM535), we
326 demonstrate diverse sensitivity to the CDK4/6 inhibitor abemaciclib (IC_{50} 0.57–4.17 μM, Emax
327 38.8–71.99%) **(Figure 6C, Supplementary Table 13)**. Activity was greatest in CAM277 (KRAS
328 ecDNA, IC_{50} 0.78 μM, Emax 71.99%) and CAM535 (CDK6-amplified 0.80 μM, 60.37%),
329 intermediate in the CDK6-amplified PDO CAM429 (0.57 μM, 49.14%), and weakest in CAM401
330 (KRAS nuclear amplified; 4.17 μM, 38.8%) exemplifying the importance of being able to match the
331 PDO specific genomic profile with the therapy response. As outlined in **Figure 6D**, individual PDO
332 sensitivity to different forms of cytotoxic chemotherapy and to EBRT varied. Overall,
333 chemotherapy sensitivity did not predict radiotherapy sensitivity.
334
335 **DISCUSSION**
336 Inter- and intra- lesional heterogeneity are fundamental, hard-to-study barriers to the effective
337 prevention and treatment of OAC[13]. Here, we derive and comprehensively characterise a biobank
338 of 116 PDOs and sc-organoids that recapitulate disease heterogeneity in BO and OAC, including
339 within lesions as well as across patient populations. As summarised in **Table 1,** we demonstrate
340 that the specific position each PDO has in the malignant spectrum cannot be discerned from a

single feature and nor can it be assumed from the characteristics of the matching tissue due to the extreme heterogeneity. Instead, the functionally relevant disease state of a given PDO can be deduced from a multi-level assessment of genomic, transcriptomic, protein and morphological characteristics **(Supplementary Figure 14)**. In this manuscript we also provide tools for supporting the quantitative characterisation of PDOs at the molecular and phenotypic level, and demonstrate that sc-organoids help capture subclonal heterogeneity and allow for the maintenance of high-risk populations that are otherwise lost over serial passages. Further, we show that individuals PDOs demonstrate variable sensitivity to EBRT and different forms of systemic anti-cancer therapy, and that responses to these treatments vary across the PDO population. In so doing, we demonstrate the utility of PDOs for studying high-risk disease features in the context of premalignant disease, as well as for analysing variable treatment responses in cancer.

Previous attempts to model BO neoplastic progression have relied on animal models and xenografts that poorly recapitulate the anatomical and biological features of human disease[55]. PDOs are in theory more scalable and have been used to study carcinogenesis in other contexts, such as for colorectal cancer[56, 57]. However, there is a long recognised challenge to establishing and propagating BO PDO cultures, with reported success rates for establishing BO PDOs of between 0-70%[55]. Here, we demonstrate derivation of 21 PDOs from regions of non-dysplastic and dysplastic BO with success rates of 68% and 61%, respectively. All remained viable over multiple passages, and all were successfully recovered from cryopreservation. This contrasts with our previous unsuccessful attempts to derive BO PDOs[16], which likely reflects the addition to culture medium of gastrin and FGF10[55].

There is also uncertainty as to the degree to which PDOs can be used to study BO dysplastic progression. Previous attempts to develop a model for OAC carcinogenesis have primarily focussed on gene editing of BO or normal gastroesophageal junctional PDOs[58, 59]. However, genetic manipulation in this context is inefficient and poor survival rates result in selection and propagation of monoclonal populations[58, 59]. Our work and that of others has also shown that OAC very likely always derives from BO[5, 60, 61], which questions the relevance of modelling dysplastic progression using normal gastroesophageal junctional PDOs. To this end, studies in colorectal cancer have demonstrated that modelling tumourigenesis using normal tissue PDOs as a starting point fails to capture many of the known molecular changes that accompany the development of

374 invasive disease[56, 57]. There is therefore a need to model progression using PDOs derived from
375 premalignant tissue.
376
377 Our comprehensive genomic, transcriptomic and phenotypic characterisation of BO PDO
378 cultures demonstrates that whilst PDOs derived from dysplastic tissue exhibited more cancer-
379 like genomic alterations than those derived from non-dysplastic tissue, they have lower
380 concordance with matching tissue. Concordance with matching tissue was also generally lower
381 for BO than it was for OAC PDOs. This is likely to be due to the nature of heterogeneity in BO, which
382 is characterised by the presence of distinct clones that do not sweep the entire segment and
383 instead arise through clonal expansion that occurs on a scale of millimetres[7, 62].
384
385 We reasoned that it is also possible that high risk subclonal populations may not be adequately
386 reflected in bulk PDO sequencing and may be lost over time or through passaging. To counter
387 this, we demonstrate a technique to isolate sc-organoids, which we show can isolate high risk
388 subclones that may not be represented in bulk PDO sequencing. These can then serve as
389 observable functional proxies for intralesional heterogeneity at a single clone level, with each
390 demonstrating varying degrees of malignant potential. Integrating these clones into the
391 epithelial–fibroblast assembloid system we describe here would enable testing of whether
392 specific high-risk lineages differentially polarise fibroblasts under pathological cues that, in turn,
393 promote their expansion and drive disease progression. Looking ahead, incorporating immune
394 cells, such as autologous or allogeneic tissue-resident myeloid or T-cell populations, and
395 immunogenic cytokines into this platform would allow interrogation of how epithelial and stromal
396 evolution interfaces with emerging immune microenvironments during early cancer development
397 and progression.
398
399 As well as being able to recapitulate the substantial heterogeneity in OAC organoids, we also
400 show that we can recapitulate the diverse treatment responses seen in patients with OAC,
401 demonstrating highly variable responses to each of the individual components of the standard
402 preoperative regimen, FLOT[14]. There is, for example, consistently high sensitivity (i.e. low IC_{50}
403 values) to docetaxel, though this has a variable maximum effect (i.e. E_{max}). In contrast, PDOs have
404 a lower and more variable sensitivity to 5-FU/leucovorin, though this consistently exerts a high
405 maximal effect when added to PDOs at a sufficiently high concentration. This mirrors clinical
406 data, where 5FU/leucovorin is the backbone of most chemotherapy regimens in OAC due to a
407 conserved effect across the entire patient population[63]. It is similarly interesting that

408 chemotherapy sensitivity does not mirror radiation or CDK inhibitor sensitivity, which provides
409 biological rationale for an expanding portfolio of studies that are combining intensified
410 preoperative chemotherapy with chemoradiotherapy or targeted agents. It would be of interest to
411 determine whether the application of the PDOs described here to an organ-on-chip approach,
412 such as that described by *Pal et al*[17], would further improve modelling of therapeutic response.
413
414 In summary, we have developed a comprehensively characterised biobank of BO and OAC PDOs
415 that reflect known disease heterogeneity across pre-malignant and invasive disease. Careful
416 characterisation is essential to understand the providence of a given organoid and derivation of
417 sc-organoids allows for the analysis of high-risk clones. Together, these models provide a
418 scalable platform for functional studies of OAC carcinogenesis and therapeutic response.
419

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435 University of Cambridge.
436

437 **AUTHOR CONTRIBUTIONS**

438 R.C.F., C.M.J. and L.Z. conceived the manuscript, obtained funding and oversaw the work. C.M.J.
439 conceived therapeutic analyses in OAC PDOs. L.Z. conceived studies relating to Barrett
440 oesophagus PDOs, single cell-derived clonal organoid and assembloid. D.H.J., G.D. and SG.J.
441 conducted bioinformatic and computational analyses. D.P.M., C.C., T.S.C., H.C., X.L., A.M., C.M.J.

442 and L.Z. performed wet laboratory experiments and led or contributed to experimental design.
443 E.B. and B.H. conducted the in-silico analysis of organoid morphologies. K.T.M., M.dP. and K.S-P.
444 contributed to sample collection, curation and characterisation. D.H.J., D.P.M., R.C.F., C.M.J. and
445 L.Z. wrote the first draft of the manuscript. All authors contributed to subsequent revisions and
446 all have read and approved the manuscript.
447
448 **DECLARATION OF INTERESTS**
449 R.C.F. is a co-founder and shareholder in Cyted Health, sits on the advisory board for AstraZeneca
450 and CRUK Functional Genomics Centre, and consults for AstraZeneca and 23andMe. C.M.J. has
451 received consultancy fees from Candesic for work outside the scope of this manuscript. M.dP.
452 received consultancy from Medtronic and Olympus for work outside the scope of this
453 manuscript. SG.J. is currently an employee of MedGenome Labs Private Ltd., Chennai, India. X.L.
454 is currently an employee of AstraZeneca, Cambridge, UK. C.C. is currently affiliated with
455 Wellcome Sanger Institute, Cambridge, UK. T.S.C. is currently affiliated with King's College
456 London, London, UK.
457
458 **METHODS**
459 **Study approach and regulatory approval**
460 Samples were obtained from surgical or endoscopic resection specimens obtained from patients
461 with known BO, dysplasia or OAC (sampling and PDO biobank summarised in **Supplementary**
462 **Figure 1)**. Prior ethical approval was provided by the United Kingdom Health Research Authority,
463 including: "A study to investigate the cell biological determinants of the development and
464 progression of the pre-malignant condition in Barrett's oesophagus" (Biomarker), 01/149;
465 "Multicentre Study to determine predictive and prognostic biomarkers and therapeutic targets
466 for oesophageal and junctional adenocarcinoma including whole genome sequencing"
467 (OCCAMS), 10/H0305/1; "A study using tissue to develop stem cell-based therapies and to
468 investigate human physiology, pathology and embryonic development" (Organ donor),
469 15/EE/0152. All patients provided written informed consent prior to sampling, which was
470 performed within a National Health Service setting.
471
472 **Sampling strategy**
473 To derive non-dysplastic BO PDOs two to three biopsy specimens were taken from the same
474 region at surveillance endoscopy, with a preference for adjacent sampling wherever possible. All
475 dysplastic BO PDOs and a single OAC PDO were obtained from specimens taken via a punch

476 biopsy from an endoscopic mucosal resection (EMR). For OAC models, resection specimens
477 were evaluated and samples obtained for organoid derivation and adjacent tissue for snap
478 freezing. For control gastric PDOs and intestinal PDOs, both patients and disease-free organ
479 donor tissues were used. Samples were briefly minced and mixed to ensure homogeneity, and
480 half or two thirds of the tissue mixture was immediately processed to derive PDOs, the methods
481 for which are outlined below. The remnant was snap-frozen using liquid nitrogen and stored at -
482 80°C for profiling of adjacent tissues recognising that given the heterogeneity there will be
483 potential differences at the cell and molecular level. To further mitigate and understand the
484 genotype to phenotype correlation in all cases, a section was cut from each snap frozen sample
485 and stained with haematoxylin and eosin (H&E) prior to DNA extraction. The H&E-stained slide
486 was independently reviewed by at least two experienced pathologists to assess cellularity, and
487 to confirm malignancy for OAC or to assess for the presence and severity of dysplasia in BO. In all
488 instances, only samples with at least 70% cellularity were used for deoxyribonucleic acid (DNA)
489 or ribonucleic acid (RNA) extraction using the AllPrep kit (Qiagen). DNA was extracted from blood
490 samples using the QIAmp DNA Blood Maxi kit (Qiagen). In all cases a germline reference was
491 obtained from blood wherever possible or otherwise from distant normal mucosa.
492
493 **Derivation of BO PDOs**
494 To derive BO PDOs, biopsy samples were rinsed in ice-cold phosphate buffered saline (PBS) and
495 pooled. These were then minced and digested using recombinant TrypLE Express Enzyme
496 (ThermoFisher) supplemented with 10μM Y-27632 (Tocris Bioscience) at 37°C for up to 10
497 minutes. Vigorous shaking was used every three minutes to dissociate tissue aggregates. Cells
498 were pelleted at 300g for 5 minutes and resuspended in 150μl TrpLE Express Enzyme. Further
499 mechanical dissociation was achieved using a P200 pipette prior to centrifugation at 300g for 5
500 minutes. The resultant single cell mixture was resuspended in Cultrex Basement Membrane
501 Extract (BME) Type 2 (R&D Systems) and plated as 15μl domes for ongoing culture.
502
503 **Derivation of OAC PDOs**
504 Samples obtained from surgical resection specimens were processed as per our previously
505 reported protocol[16]. Briefly, each sample was rinsed in ice-cold PBS, minced then digested
506 through incubation at 37°C with collagenase II (1.5mg/ml) for up to 2 hours. Large fragments were
507 then removed by filtration using a 70μM cell strainer and the resultant cell suspension centrifuged
508 at 400g for 2 minutes. The pellet was resuspended in PBS as a further wash step to remove debris

509 and a further identical centrifugation step performed. Isolated cells were then resuspended in
510 Cultrex BME Type 2 and plated as 15µl-20µl domes for ongoing culture.
511
512 **Derivation of gastric and intestinal PDOs**
513 Gastric PDOs were established either from endoscopic biopsies of the gastric cardia from BO
514 patients or from gastroesophageal junction (GOJ) tissue from disease-free organ donors. For
515 gastric cardia PDOs derived from BO patients, biopsy specimens were processed using the same
516 protocol as for BO PDO derivation. Gastric PDOs from organ donors were generated as described
517 in our previous study[5] . Briefly, a circumferential tissue fragment (up to 10 cm in length) spanning
518 3–5 cm of distal oesophagus and 3–5 cm of proximal stomach was obtained. GOJ PDOs were
519 established from a 5 mm tissue strip spanning the Z-line (squamous–columnar junction). Gastric
520 body PDOs were derived from tissue located at least 2 cm below the Z-line. Intestinal PDOs were
521 kindly provided by Professor Fiona Gribble, University of Cambridge. In brief, intestinal organoids
522 were generated from healthy jejunal or duodenal tissue obtained from patients undergoing
523 gastrectomy or pancreaticoduodenectomy **(Supplementary Table 2)**. Note: although organ-
524 donor-derived organoids are not strictly patient-derived, we refer to them as PDOs for
525 consistency and ease of interpretation throughout the manuscript.
526
527 **PDO culture**
528 All PDO cultures were maintained in a humidified atmosphere with 5% carbon dioxide (CO_2) at
529 37°C. PDOs were maintained in IntestiCult™ Organoid Growth Medium (StemCell Technologies)
530 supplemented with Primocin (1mg/ml, InvivoGen) and Y-27632 (10µM), with or without FGF10
531 (100ng/ml, Peprotech) and Gastrin (10nM, Sigma). Media was refreshed every two to five days for
532 cell maintenance outside of specific experimental conditions. PDOs were passaged at least
533 every two weeks through the addition of pre-warmed TypLE Express Enzyme and manual
534 dissociation by pipetting. The resultant cell solution was centrifuged at 300g for 5 minutes,
535 providing a cell pellet that was resuspended in Cultrex Basement Membrane Extract, Type 2 and
536 plated as 15µl-20µl domes that were allowed to polymerise prior to the addition of media. Once
537 established, BO and intestinal PDO cultures were maintained in organoid medium (OWR)
538 prepared from Advanced Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with
539 0.5nM Wnt Surrogate-Fc Fusion Protein (N001, U-Protein Express BV), 9.6nM RSPO3-Fc Fusion
540 Protein conditioned medium (R001, U-Protein Express), 10mM HEPES, 1× Glutamax, 1× N2,
541 100ng/mL Noggin, 10 mM Nicotinamide, 1 mM N-acetylcysteine, 1×B27, 50ng/mL EGF, 500nM
542 A8301, 10µM SB202190, 1× Primocin, 10nM Gastrin, 10µM Y-27632 and 100ng/mL FGF10.

543

544 **Establishment of single cell-derived clonal organoids**

545 To isolate specific clonal populations, we cultured PDOs to around 70-80% confluency in Cultrex
546 BME Type 2 domes. Culture medium was then removed and replaced with an ice-cold mixture
547 comprising of a 1:1 ratio of TrypLE Express Enzyme and Advanced Dulbecco's Modified Eagle
548 Medium (DMEM)/F-12 supplemented with 10µM Y27632 (TrypLE/ADF). A P1000 pipette was then
549 used to manually break the BME Type 2 domes whilst leaving PDOs intact. The resultant mixture
550 was transferred to the underside of a sterile Corning cell culture plate lid with multiple 40µl
551 TrypLE/ADF droplets. Using a brightfield microscope, the PDO suspension was inspected for
552 single organoids exceeding a diameter of 200µm. Once identified, these were aspirated under
553 direct vision using a P20 pipette and transferred to a TrypLE/ADF droplet. This process was
554 repeated multiple times and each droplet then transferred to a fresh 1.5ml microcentrifuge tube.
555 This was incubated at 37°C for five minutes using a ThermoMixer® (Eppendorf). A P20 was then
556 used to mechanically dissociate the isolated PDO and the resultant single cell solution
557 resuspended in Cultrex BME Type 2 and plated as 15µl domes for ongoing culture. Of note, to
558 study heterogeneity in BO and dysplasia, single organoids were picked from passage 0 cultures,
559 i.e. cultures established directly from tissue without any passaging, to ensure that distinct clonal
560 populations were selected.

561

562 **Establishment of assembloid co-cultures**

563 Primary human oesophageal fibroblasts (ABM-T4232, Applied Biological Materials), were a gift
564 from Professor Jacqueline Shields, University of Nottingham. Fibroblasts were maintained in
565 Fibroblast Medium (SC-2301, Caltag Medsystems Ltd). Organoids were harvested, resuspended,
566 and mixed with fibroblasts at a 1:2 ratio in OWR medium supplemented with 5% foetal bovine
567 serum (FBS) and 10% Cultrex BME Type 2, and seeded into round-bottom 96 well plates. Each
568 well contained 100µL with 225,000 cells. Plates were centrifuged at 300g for 5 minutes to pellet
569 cells and then incubated at 37°C overnight. During this period, the cells self-assembled into a
570 sphere, which was subsequently transferred into a Cultrex BME Type 2 dome in a 48 well plate as
571 assembloids and maintained in OWR medium for 7 days.

572

573 To explore microenvironmental influences, assembloids were treated for 7 days with either 5 mM
574 refluxate salt (sodium glycocholate hydrate; Sigma), 10 ng/mL IL10 (R&D Systems), or a
575 combination of both. For conditions containing refluxate salt, assembloids were exposed to the
576 treatment for only 6 hours per day. Assembloids from each treatment condition were dissociated

577 into a single cell suspension and stained with LIVE/DEAD™ Fixable Violet (1:1000, Thermo Fisher
578 Scientific). For detection of human-specific markers, the following antibodies were used: anti-
579 EpCAM (APC, clone 9C4, BioLegend), anti-α-SMA (PE, clone 1A4, R&D Systems), and anti-CD34
580 (BV510, clone 581, BioLegend). Intracellular staining was performed as outlined previously[21].
581 Briefly, cells were fixed using the Cytofix/Cytoperm Fixation and Permeabilization Solution (BD
582 Biosciences, UK) and permeabilized with 1x permeabilization buffer (Thermo Fisher Scientific,
583 UK). Positive staining was assessed relative to fluorescence-minus-one (FMO) controls. Doublets
584 and dead cells were excluded during analysis. Fibroblasts within the assembloids were gated
585 from the EpCAM-negative population. Data were acquired on an LSRFortessa (BD Biosciences)
586 and analysed using FlowJo software (Freestar Inc).
587
588 **PDO treatment using systemic anti-cancer therapies**
589 The viability of OAC PDOs was assessed following exposure to individual components of FLOT (all
590 supplied by SelleckChem) and to the cell cycle inhibitor, abemaciclib (LKT labs). To mirror clinical
591 administration, oxaliplatin and docetaxel were administered to PDOs as single agents whereas
592 5-fluorouracil was administered in combination with a fixed dose of leucovorin. Each drug was
593 reconstituted as per manufacturer instructions and each was diluted in either DMSO (5-
594 fluorouracil, docetaxel, and abemaciclib) or ultrapure water (oxaliplatin and leucovorin) to values
595 within a range of 200μM-0.195μM for components of FLOT, except for leucovorin, which was
596 administered at 10μM on all occasions, and 0.001-10μM for abemaciclib. Values for both dilution
597 series were selected based on others reported in the literature[22, 23]. The concentration of DMSO
598 was fixed at ≤0.1% within treatment and control vehicle wells.
599
600 In line with existing protocols, PDOs were dissociated using TrypLE Express Enzyme then counted
601 and resuspended in BME Type 2 prior to drug exposure[16, 24]. A total of 5000 cells were seeded
602 within 20μL BME Type 2 droplets formed at the centre of each well of a 96-well plate. These were
603 allowed to polymerise prior to the addition of media. Cultures were maintained for 72 hours and
604 then exposed to drug when in a logarithmic growth stage. Viability was assessed after 144 hours
605 exposure to components of FLOT, abemaciclib, or vehicle. Media, including drug or vehicle, was
606 refreshed after 72 hours for PDOs treated with FLOT constituent drugs.
607
608 **PDO treatment using external beam radiotherapy**
609 Prior to irradiation, established PDOs were dissociated to a single cell solution. This was
610 performed by first dissociating PDOs from Cultrex Basement Membrane Extract, Type 2, through

611 incubation with pre-warmed TypLE Express Enzyme and manual agitation by pipetting.
612 Dissociated PDOs were pelleted by centrifugation at 300g for 5 minutes and resuspended in 30µl
613 cool PBS. A P20 pipette was then used to manually dissociate the PDOs to a single cell solution
614 and the cell concentration assessed using a Countess II Automated Cell Counter (Invitrogen). A
615 total of 10,000 cells were plated in 15µl Cultrex Basement Membrane Extract, Type 2 with media
616 added once this had polymerised. The media was replaced with pre-warmed PBS prior to
617 irradiation 24 hours later using a CIX1 (XStrahl) benchtop irradiator. Single fraction treatments of
618 0Gy (sham), 2Gy, 4Gy, 6Gy and 8Gy x-ray irradiation were delivered and PBS then replaced with
619 media 15 minutes after irradiation.
620
621 **Whole genome sequencing**
622 Extracted DNA was subjected to paired-end whole genome sequencing at a depth of 50x for
623 tissues, 30x for PDOs and 15x for sc-organoids, using a well-established pipeline[11]. A
624 combination of in-house tools and FastQC
625 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) were used for quality control.
626 Sequencing reads were aligned against the hg19/Ensembl GRCh38 reference genome for
627 mutation calling using the Burrows-Wheeler BWA-MEM alignment algorithm[25]. Aligned reads
628 were subsequently sorted into genome coordinate order and Picard
629 (http://broadinstitute.github.io/picard) used to remove duplicate reads. Single nucleotide
630 variants and insertion deletion mutaitons were called with Strelka v2.0.15[26]. Tumour purity and
631 ploidy were inferred with ASCAT-NGS v2.17, and copy number alterations derived with ASCAT
632 from read counts at germline heterozygous sites identified by GATK HaplotypeCaller v3.2-2,
633 adjusting for the estimated normal-cell component. Structural variants (SVs) were called using
634 MANTA v0.27 with any SVs that had any supporting reads in the matched normal being discarded
635 as previously described[27].
636
637 **RNA sequencing**
638 Extracted RNA was quality assessed using the RNA 6000 Nano Kit (Agilent) on a 2100 Bioanalyser
639 (Agilent). Samples for which there was sufficient material available for further analysis and that
640 had a calculable RNA integrity number were determined to have passed quality control and were
641 quantified using the Qubit High sensitivity RNA assay kit (Thermo Fisher). The TruSeq Stranded
642 Total RNA High Sensitivity protocol with ribosomal depletion was used to prepare libraries using
643 an input of at least 100ng but preferably 250ng if available. KAPA quantification (KAPA
644 Biosystems) was used to quantify libraries, which were pooled and run on a HiSeq 4000 to

645 generate 75 base pair adjacent-end reads. After sequencing, reads were aligned by STAR. Gene-
646 level counts for each sample were generated using Bioconductor's GenomicAlignments package
647 together with ENSEMBL gene annotations. These raw counts, along with sample-specific
648 sequencing depths and gene lengths, were then used to calculate Transcripts Per Kilobase Million
649 (TPM) values for individual genes across all samples.
650
651 **Bioinformatic analyses**
652 In determining relevant driver events, we considered mutations in 76 genes identified by Frankell
653 et al. as positively selected for in OAC[11]. Amplification events were defined as cases where copy
654 number was more than double the sample ploidy, whilst deletion events were strictly defined as
655 a total copy number of zero. Losses in the single cell-derived clonal organoids were defined as a
656 minor copy number of zero. Two PDOs (OESO/117 and OESO/146) were used for chemotherapy
657 treatment screens but underwent WGS elsewhere. Clinical and genomic information for these
658 PDOs was obtained from Cell Model Passports (https://cellmodelpassports.sanger.ac.uk/).
659
660 The HRD index score, the sum of three HRD-associated copy number features applied here as a
661 broad measure of total chromosomal instability, was calculated using the scarHRD R package[28,
662 29]. Mutational signatures were extracted de novo using SigProfiler applied to the complete set of
663 OAC tissues and PDOs, resulting in the following signatures: SBS1, SBS2, SBS5, SBS8, SBS13,
664 SBS17a, SBS17b, SBS18, SBS31, SBS35, SBS40, SBS93[30]. These signatures were then fitted using
665 the deconstructSigs R package with a 2% prevalence cut-off[31]. Signatures SBS31 and SBS35 were
666 not fitted for BO PDOs since they are caused by platinum chemotherapy. Subclonal
667 reconstruction of the non-dysplastic BO PDOs was conducted with PyClone-VI using the beta-
668 binomial distribution[32].
669
670 Phylogenetic analysis of the single cell-derived clonal organoids was conducted using the
671 parsimony ratchet method as implemented through the phangorn R package[33], with branch
672 lengths assigned according to the ACCTRAN criteria. Specific SNVs were assigned to branches
673 onto which they fitted exactly based on their presence across organoids deriving from the
674 respective branch. A subset of the above signatures (SBS1, SBS2, SBS5, SBS8, SBS13, SBS17a,
675 SBS17b, SBS18, SBS40) with established presence in BO, and that do not relate to chemotherapy
676 treatment, were fitted using deconstructSigs to any branch with more than 25 unique SNVs.
677 Barplots for signature contributions were plotted onto the corresponding branch and extended
678 manually to fit the branch length.

679

680 Differential gene expression analysis was conducted using the DESeq2 R package[34]. Functional
681 enrichment of differentially expressed gene sets was analysed using the gProfiler2 R package[35],
682 specifically on genes with p-value (false discovery rate adjusted for multiple testing) of less than
683 0.05 and with an absolute log2-fold change greater than 1, and separated into up- and down-
684 regulated genes. This package enables enrichment analysis against multiple databases including
685 Gene Ontology, the Human Protein Atlas for cell-type analysis, and TRANSFAC for transcription
686 factor-binding profiles. Gastric and intestinal gene set 'scores' were calculated through gene set
687 variation analysis of the TPM-normalised data, using the GSVA R package[36], taking *MUC6*,
688 *MUC5AC*, *CLDN18*, *PGC*, *GKN1*, *GKN2* as gastric genes and *MUC2*, *TFF3*, *REG4*, *GPA33*, *CDX2*,
689 *FABP2*, *OLFM4*, *ANPEP* as intestinal genes.

690

691 RNA-seq counts for OAC-derived PDOs underwent batch correction using ComBat, as part of the
692 sva R package[37]. Principal component analysis was conducted on the top 1000 variable genes
693 following batch correction and variance stabilising transformation. Gene set enrichment
694 analysis, using the cancer hallmark gene sets, was applied on the loadings of each gene for the
695 first two principal components using fgsea. Normal gastric, normal intestinal, and BO-derived
696 PDOs were sequenced in the same batch and so correction was not required.

697

698 **Organoid forming efficiency assay**

699 All PDOs were imaged on the day of, and 10-days following, irradiation using an Incucyte® SX5
700 Live-Cell Analysis System (Sartorius, Goettingen, Germany). The Incucyte® Organoid Analysis
701 Module (Sartorius, Goettingen, Germany) was used to count the number of PDOs at each
702 assessed timepoint using a segmentation radius of 100µm, segmentation sensitivity of 30,
703 segmentation edge split with sensitivity of 100, cleanup with hole fill of 500,000µm^2 and an
704 adjusted pixel size of -1. Filters were set to count objects as PDOs only with a minimum area of
705 1500µm^2 and maximum eccentricity of 0.25 as a strict cut-off to exclude budding, migrating or
706 necrotic PDOs. Given that there should not be PDOs formed at day zero, the PDO count at this
707 point was considered to reflect noise and was subtracted from the day 10 PDO count. Organoid
708 formation efficiency was calculated for each radiation dose as the number of formed PDOs at
709 day 10 divided by the cell seeding number, 10,000. Relative organoid forming efficiency was
710 determined by dividing the organoid forming efficiency at each delivered dose by the organoid
711 forming efficiency at 0Gy.

712

713 **PDO viability**

714 PDO viability was quantified using a luminescent assay 72- or 144- hours after respective
715 administration of abemaciclib or FLOT. In accordance with manufacturer instructions, 20uL of
716 CellTiter-Glo® 3D (Promega) was applied to each well of a 96-well plate, which was then shaken
717 for five minutes. Plates were then incubated for 30 minutes in a humidified atmosphere with 5%
718 CO_2 and luminescence read using an Infinite 200 Pro microplate reader (Tecan).

719

720 **PDO fluorescent labelling**

721 PDOs derived from BO and OAC were labelled with fluorescent nuclear markers via lentiviral
722 transduction. Briefly, PGK-H2B-eGFP or PGK-H2B-mCherry plasmids were co-transfected with
723 pMD2.G and psPAX2 into HEK293 cells (a gift from Sakari Vanharanta, University of Helsinki) to
724 produce lentivirus, following the protocol described in Kita-Matsuo et al[38]. Viral supernatant was
725 concentrated 16-fold using the Lenti-X™ Concentrator (631231, Takara Bio) and resuspended in
726 organoid medium. Organoids were harvested, resuspended in 15 µL (approximately 500,000
727 cells), mixed with 250 µL of concentrated lentivirus, and incubated for 4 hours at 37 °C. The
728 organoids were then washed, seeded, and maintained under standard culture conditions.

729

730 PGK-H2BeGFP was a gift from Mark Mercola (Addgene plasmid # 21210;
731 http://n2t.net/addgene:21210; RRID: Addgene_21210). PGK-H2BmCherry was a gift from Mark
732 Mercola (Addgene plasmid # 21217; http://n2t.net/addgene:21217; RRID: Addgene_21217).
733 pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259; http://n2t.net/addgene:12259;
734 RRID: Addgene_12259). psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260;
735 http://n2t.net/addgene:12260; RRID: Addgene_12260).

736

737 **Fluorescent in situ hybridisation**

738 Flourescent *in situ* hybridisation (FISH) was performed using routine diagnostic *HER2* probes. As
739 previously described[39], PDO cultures were prepared using standard cytogenic procedures for
740 harvesting, fixation (3:1 methanol : acetic acid solution) and slide formation. All subsequent pre-
741 treatment and hybridisation steps were undertaken in accordance with manufacturer
742 instructions by the Department of Histopathology, Cambridge University Hospitals NHS
743 Foundation Trust.

744

745 **Time-lapse imaging of PDO growth and morphology**

Time lapse imaging was performed using a Zeiss Axio Observer Live Cell Station at 10x magnification at either 2-4 hourly intervals or at day 4, 6 and 7 after single cell seeding. Up to four locations were imaged at each time interval with images captured across up to 23 slices through a z-stack of the Cultrex BME Type 2 dome in which the PDOs are cultured. Brightfield images were acquired, in addition to GFP fluorescence via excitation at 469nm with emission recorded at 525nm using a GFP filter set, and mCherry fluorescence via excitation at 590nm with emission at 687nm using an mCherry filter set. Exposure time and acquisition parameters were kept constant at all imaging intervals. Images were processed using Zeiss Zen software.

Computational analyses of PDO growth kinetics and morphology

Once processed, images of PDOs obtained at each timepoint were analysed using the Fiji distribution of ImageJ[40, 41]. Given an inherent partial eccentricity, each PDO was manually delineated using an ellipse. The minimum and maximum widths of this were averaged to give an approximate value for the PDO diameter. Though often visible across multiple levels of a z-stack, PDOs were measured only once, at the plane at which they were most in focus. PDOs were simultaneously categorised by morphology and distinguished by the presence or absence of a visible lumen, which if present was categorised as single or multiple, and whether the cells surrounding the lumen were a single cell layer or of multiple layers. For hollow PDOs (i.e. those with a lumen), the number of cells within that PDO was presumed to be proportional to the square of the diameter (i.e. the surface area). For solid organoids (i.e. those lacking a lumen), the number of cells in that PDO was presumed to be proportional to the cube of the PDO (i.e. the volume of the organoid).

An *in silico* cell-level model of PDO growth was built in CompuCell3D (CC3D) Version 4.2.5[42]. This implements a lattice-based multi-site Cellular Potts Monte Carlo model and has previously been used to model cells growing around a lumen. Briefly, each cell in this model can occupy a number of lattice sites. A model is developed to minimise the energy of the lattice conformation using the following equation:

$$E = \sum_{\vec{i},\vec{j}} J\left(\tau\left(\sigma(\vec{i})\right), \tau\left(\sigma(\vec{j})\right)\right)\left(1 - \delta\left(\sigma(\vec{i}), \sigma(\vec{j})\right)\right) + \sum_{\sigma} \lambda_{vol}(\sigma)\left(v(\sigma) - V_t(\sigma)\right)^2$$

where the first sum represents energy changes due to cell adhesion for every pair of neighbour lattice sites (*i, j)* and the second sum represents volume constraints for every cell, σ. In the first sum, *J* represents different adhesion energies between each pair of cell types $\tau\left(\sigma(\vec{i})\right)$ and

$\tau(\sigma(\vec{j}))$, or between a cell type and the extracellular matrix. In the second equation, each cell has a target volume $V_t(\sigma)$ and a current volume $v(\sigma)$. The energy equation penalises deviation from the target volume, with $\lambda_{vol}(\sigma)$ representing cell elasticity. Multiple Monte Carlo Steps occur, with each considered a time unit proportional to experimental time. At each step each pixel will attempt to copy itself to a neighbouring lattice site. This will succeed with probability 1 if the change reduces the overall energy, E, in the system.

The model was implemented in two dimensions, representing experimentally captured images of PDOs in cross section. Three cell types were used: polar, non-polar and lumen. Each lumen was modelled as a single 'cell', with the size of this lumen 'cell' determined by the number and type of cells surrounding the lumen.

Statistical analysis and reproducibility

All experiments were undertaken in at least biological triplicate unless otherwise stated. Given the known challenges of inter-batch heterogeneity in Cultrex BME Type 2 (R&D Systems), all biological replicates for irradiation experiments were performed using the same batch of BME Type 2. Integral area under the curve (AUC) values for organoid forming efficiency following irradiation were calculated using GraphPad Prism Version 10.4.1. for Windows (GraphPad Software, Boston, USA). Percent viability following exposure to systemic anti-cancer therapy was normalised to vehicle control and modelled against drug concentration using a three-parameter logistic (3PL) curve in GraphPad Prism v10.4.1 with Top fixed at 100%, and Bottom and Hill slope free. A four-parameter logistic (4PL) was also fit and retained only if it was clearly supported by the Akaike Information Criterion with small-sample correction (AICc) (ΔAICc ≤ -4) and the top 95% confidence interval excluded 100% across biological replicates; otherwise 3PL was used. Emax is reported as 100 − Bottom and the area under the curve (AUC) was computed over the tested log-dose range and normalised and reported as 1-AUC. Pairwise comparisons were made using the Mann-Whitney U test unless otherwise stated. Comparisons of more than two groups were made using the Kruskal Wallis test unless otherwise stated. Adjustments for multiple significance testing were made using the Bonferroni test unless otherwise stated in instances in which repeated statistical comparisons were drawn from the same dataset.

Data availability

Metadata to correlate information within this archive to that published here is provided in **Supplementary Table 14**.

Code availability

The scripts developed during the analysis presented here are available at the following GitHub repository, released under a GNU GPL-v3.0 license: https://github.com/fitzgerald-lab/OAC_Barretts_PDOprofiling. This includes scripts for analysing WGS data (e.g. driver gene profiles, mutational concordance, signature contributions, phylogenetic analysis, clonal dynamic analysis) and RNA-seq data (differential expression, unsupervised clustering, pathway and gene-set analysis) obtained from BO, normal tissue-, and OAC-derived PDOs, and sc-organoids.

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1009 **LIST OF FIGURES**

1010 **Figure 1: The genomic landscape of normal gastric and Barrett oesophagus-derived PDOs**
1011 **and its correlation with parent tissue samples. (A)** Driver alterations and genomic features
1012 across PDOs and adjacent tissues. Driver genes were considered according to the set of genes
1013 (n=76) found to be positively selected in OAC by Frankell et al[11] **(B)** Concordance of mutational
1014 profiles across Barrett oesophagus tissue-PDO pairs, defined by the proportion of mutations
1015 shared and unique across samples. Tissues from normal stomach did not undergo WGS. **(C-D)**
1016 Comparisons of **(C)** indel and SNV burdens, and **(D)** ploidy across tissue-PDO pairs. **(E)**
1017 Comparisons of mutational signature profiles across tissue-PDO pairs calculated by cosine
1018 similarity. **(F)** Correlations in SBS17a (left) and SBS17b (right) proportions across tissue-PDO
1019 pairs. Pearson correlation coefficients were used to determine correlation strengths.

1020

1021 **Figure 2: Diversification of non-dysplastic Barrett oesophagus demonstrated in a single cell-**
1022 **derived clonal organoid panel. (A)** Approach for establishing sc-organoids. **(B)** Distributions of
1023 VAFs from single cell-derived organoids (n=19) derived from AHM2149. **(C)** Driver alterations
1024 found across the single cell-derived clonal organoid panel and adjacent bulk PDO and tissue. **(D)**
1025 A phylogenetic profile of organoids derived from AHM2149 defined using the sc-organoid panel.
1026 Signature contributions were calculated across mutations contributing to each branch
1027 respectively. Branch lengths are generally proportional to total amount of mutations. Signatures
1028 were not fitted for branches with fewer than 25 SNVs. **(E)** Hierarchical clustering of RNA-seq
1029 profiling for the sc-organoid panel using the top 500 variable genes following variance stabilised
1030 transformation of read counts. Heatmap annotation includes genetic alterations as reported in
1031 **(C)** and log2-transformed TPM-normalised counts of *MUC6* (a gastric marker) and *CDX2* (an
1032 intestinal markers), the correlation of which is reported in Supplementary Figure 6E. The coloured
1033 dashed squares found in **(D)** and **(E)** correspond to adjacent clones found through phylogenetic
1034 analysis and unsupervised clustering of RNA-seq.

1035

1036 **Figure 3: Analysis of key morphological features observed in Barrett oesophagus PDOs. (A)**
1037 Representative bright-field images showing the four morphology classes: a single cell layer
1038 surrounding a single lumen, a single cell layer with multiple lumens, multiple cell layers with a
1039 lumen and a solid morphology with no visible lumen. In all instances the red scale bar represents
1040 a length of 100μm. **(B)** Example field of view illustrating the coexistence of multiple morphologies
1041 within a single dome of non-dysplastic (AHM0040) Barrett oesophagus PDOs. Scale bar
1042 represents 200μm. **(C)** Stacked bar plot of the count of each morphology class over seven days

of growth following seeding in non-dysplastic (AHM0040) PDOs. **(D)** Proportional composition of morphologies at the indicated time points (normalised to total organoids measured per day) for dysplastic (AHM2221) PDOs. **(E)** Diameter squared (proportional to number of cells) of non-dysplastic (AHM0040) and dysplastic (AHM2221) PDOs at days 4 and 6 following seeding. **(F)** *In silico* simulations of organoid growth/morphology across four random seeds under three scenarios depicting variable likelihood of inheriting polarity (IP).

Figure 3: Transcriptomic profiling of control normal gastric and intestinal, and Barrett oesophagus, PDOs. (A) Inter-PDO expression of gastric- and intestinal-related genes. The reported scores were calculated using Z-normalisation following log2-transformation of TPM values. **(B)** Principal component analysis of all normal- and Barrett oesophagus PDOs based on the top 500 variable genes following variance stabilising transformation. **(C)** Correlations of log2-fold changes from differential expression analysis on non-dysplastic Barrett versus normal gastric, and dysplastic Barrett versus normal gastric PDOs. Genes were considered significant if they had an adjusted p-value < 0.05 and absolute log2-fold change > 1, as indicated by the dashed red lines. Full results of these analyses are reported in **Supplementary Figure 5D-E**. **(D)** Differential expression across dysplastic (n=5) versus non-dysplastic (n=9) Barrett oesophagus PDOs. Positive log2-fold change refers to increased expression in the dysplastic PDOs. Genes were coloured based on their adjusted p-value being less than 0.05, and absolute log2-fold change greater than 1. **(E)** Functional enrichment analysis of up- and down-regulated genes shared across non-dysplastic and dysplastic PDOs compared with normal gastric PDOs. Full functional enrichment analysis results are reported in **Supplementary Table 6**. **(F)** Expression of *HOXB2*, *HOXB6*, and *HOXB8* across all control normal and Barrett oesophagus PDOs. Values reported are log2-transformed TPM values, and comparison was done on the non-dysplastic and dysplastic PDOs using a two-sided Wilcoxon test with Benjamini-Hochberg adjustment for multiple testing.

Figure 5: An OAC PDO biobank captures parent tissue features and recapitulates multi-level population diversity. (A) Driver alterations and genomic features across OAC-derived PDOs and adjacent tissues. Driver genes were considered according to those previously defined by Frankell et al[11]. **(B)** Concordance of mutational profiles across OAC tissue-PDO pairs, defined by the proportion of mutations that are shared or unique across samples. **(C)** Circos plots demonstrating genomic features of OAC PDOs and their parent tissues. **(D)** Comparisons of mutational signature profiles across tissue-PDO pairs calculated by cosine similarity. **(E)**

1077 Principal component analysis of bulk RNAseq profiles of derived OAC PDOs and relationship to
1078 tumour stage.
1079
1080 **Figure 6: OAC PDOs demonstrate variable responses to chemotherapy, radiotherapy and**
1081 **targeted agents. (A)** Inhibitory concentration curves demonstrating the viability of five OAC PDOs
1082 following exposure to **(i)** docetaxel, **(ii)** 5-fluorouracil (5-FU) and leucovorin, or **(iii)** oxaliplatin. **(B)**
1083 Organoid forming efficiency following exposure to a single fraction of 0-8Gy x-ray irradiation for
1084 eight OAC PDOs. **(C)** Correlation between AUC organoid forming efficiency following x-ray
1085 irradiation and AUC viability values following exposure to 5-FU and leucovorin, oxaliplatin or
1086 docetaxel. **(D)** Inhibitory concentration curves demonstrating he viability of four OAC PDOs
1087 following exposure to the CDK4/6 inhibitor, abemaciclib. **(E)** Normalised AUC sensitivity values
1088 for each anti-cancer treatment across three separated PDOs (created in https://BioRender.com).

1089 **LIST OF SUPPLEMENTARY FIGURES**

1090 **Supplementary Figure 1: Schematic overview of the 116 normal, Barrett oesophagus and**
1091 **OAC bulk PDOs and single cell-derived organoids generated for this study.** The site from
1092 which samples were obtained, and the sampling route, is shown, as is a summary of the multi-
1093 level characterisation employed for all obtained models. Image generated using BioRender®.

1094

1095 **Supplementary Figure 2: Concordance of genomic features between Barrett oesophagus**
1096 **PDOs and their parent tissues.** Tumour mutation burden across **(A)** PDOs derived from normal
1097 gastric and BO tissue, and **(B**) tissues derived from non-dysplastic and dysplastic BO. **(C)**
1098 Differences in VAFs of shared mutations across PDO-tissue pairs, with the dotted red-line
1099 indicating mutations where $VAF_{organoid} - VAF_{tissue} = 0$ (i.e. where a mutation has equal VAF in a PDO
1100 and its adjacent tissue). **(D) (i)** Comparisons of tumour purity between adjacent tissues and
1101 PDOs, and **(ii)** comparisons of tumour purity between non-dysplastic and dysplastic Barrett
1102 oesophagus. Purity within PDO-tissue pairs was calculated via a pairwise Wilcoxon test, and
1103 purity across Barrett dysplastic stages was compared via a two-sample Wilcoxon test. **(E)**
1104 Comparisons of indel and SNV burdens across PDO-tissue pairs. Correlation strengths were
1105 reported as Pearson correlation coefficients. The black dotted line represents the line where (i.e.
1106 where the number of mutations is equal between a PDO and its adjacent tissue). **(F)**
1107 Comparisons of HRD index scores across PDO-tissue pairs, with Pearson correlation coefficients
1108 reported for non-dysplastic dysplastic BO derived samples. The black dotted line represents the
1109 line where $HRD_{organoid} = HRD_{tissue}$ (i.e. where the HRD index scores for a PDO is equal to
1110 that of its adjacent tissue). **(G)** Comparisons of cosine similarity scores of signature profiles as
1111 reported in **Figure 1F** and **Supplementary Figure 1D** across non-dysplastic and dysplastic
1112 Barrett oesophagus-derived PDO-tissue pairs. Similarity scores were compared using a two-
1113 sample Wilcoxon test. **(H)** Summary of mutational signature analysis on adjacent Barrett
1114 oesophagus-derived PDOs and tissues. Samples (rows) are ordered into PDO-tissue pairs. **(I)**
1115 Correlations in SBS17a/b contributions across PDO-tissue pairs with Pearson correlation
1116 coefficients calculated separately across non-dysplastic and dysplastic samples. **(J)**
1117 Comparisons of SBS17a/b contributions across non-dysplastic and dysplastic Barrett
1118 oesophagus-derived PDOs. Contributions were compared using two-sample Wilcoxon tests.
1119 Correction for multiple testing was conducted using the Benjamini-Hochberg procedure. **(K)** VAF
1120 distributions of AHM0040, a PDO derived from a non-dysplastic Barrett oesophagus case. Dotted
1121 lines represent the VAFs of mutations in OAC-associated driver genes. **(L)** VAF distributions of

1122 shared mutations at passages 1 and 6 of AHM1373, a non-dysplastic derived PDO. Colours refer
1123 to the clonal classifications of mutations at passage 1.
1124
1125 **Supplementary Figure 3: Clonal expansions of Barrett PDOs across passages and within a**
1126 **non-dysplastic Barrett-derived panel of single cell-derived clonal organoids. (A)** Tumour
1127 mutation burdens of the single cell-derived clonal organoid panel derived from a non-dysplastic
1128 Barrett oesophagus PDO (AHM2149), with mutations classified as those shared with the bulk
1129 organoid from which they were derived, those not captured in the bulk organoid but present in at
1130 least one derived organoid, and private mutations. **(B)** Signature contributions across all
1131 mutations from the single cell-derived clonal organoid panel and its adjacent tissue and bulk
1132 organoid. **(C)** Correlation between *MUC6* (gastric marker) and *CDX2* (intestinal marker).
1133
1134 **Supplementary Figure 4: Multi-omic profiling of a single cell-derived clonal organoid panel**
1135 **obtained from a dysplastic Barrett oesophagus PDO. (A)** VAF distributions across a single cell-
1136 derived clonal organoid panel (n=9) derived from a dysplastic PDO (AHM2392). Normal
1137 distributions around VAF=50% indicate clonality. **(B)** Tumour mutation burdens of the dysplastic-
1138 derived single cell-derived clonal organoid panel, with mutations classified as those shared with
1139 the bulk organoid from which they were derived, those not captured in the bulk organoid but
1140 present in at least one derived organoid, and private mutations. **(C)** Signature contributions
1141 across all mutations from the derived organoid panel, alongside adjacent tissue and bulk PDO
1142 obtained from the dysplastic and OAC tissues. **(D)** A phylogenetic profile of the PDO derived from
1143 AHM2392 defined using the derived organoid panel. Signature contributions were calculated
1144 across mutations contributing to each branch, respectively. Branch lengths are generally
1145 proportional to total amount of mutations. Signatures were not fitted for branches with fewer than
1146 25 SNVs. **(E)** Hierarchical clustering of RNA-seq profiling for the dysplasia single cell-derived
1147 clonal organoid panel using the top 500 variable genes following variance stabilised
1148 transformation of read counts. Heatmap annotation includes genetic alterations as reported in
1149 (F) and log2-transformed TPM-normalised counts of *MUC6* (a gastric marker) and *CDX2* (an
1150 intestinal markers). The green dotted lines in **(D)** and **(E)** represent matching clones according to
1151 the phylogenetic profiling and hierarchical clustering. **(F)** Driver alterations found across the
1152 dysplasia single cell-derived clonal organoid panel as well as bulk PDO and tissue derived from
1153 dysplasia and matched OAC tissue.
1154

1155 **Supplementary Figure 5: Morphological analysis of Barrett oesophagus PDOs. (A)**
1156 Representative bright-field images illustrating the three morphology classes visible in a
1157 dysplastic Barrett oesophagus PDO (AHM2221): single layer, single lumen; multiple layer, single
1158 lumen; and solid (multiple cells, no visible lumen). Lower panel shows coexistence of multiple
1159 morphologies within a single culture. Scale bars: 100 µm (top), 200 µm (bottom). **(B and C)** Size
1160 distribution of single cells identified in the non-dysplastic (AHM0040) and dysplastic (AHM2221)
1161 Barrett oesophagus PDO cultures. Histograms show counts per 1µm; dashed vertical line marks
1162 the mean cell diameter. PDOs were counted as a diameter of 21µm or greater. **(D)** Diameter
1163 distributions of measured non-dysplastic (AHM0040) PDOs stratified by morphology class and
1164 day (within each class, lighter to darker bars denote increasing time). Each subplot shows the
1165 frequency (proportion of organoids per bin) for that class/day. **(E)** Relationship between non-
1166 dysplastic (AHM0040) PDO size and proliferative activity. Boxplots show organoid diameter
1167 versus the number of nuclei scored per organoid, colour-coded by day (as labelled). Boxes
1168 indicate interquartile range, with the median value shown as the centre line. **(F)** Mean non-
1169 dysplastic (AHM0040) PDO area over time, separated by morphology (multiple lumen, blue;
1170 single lumen, red). **(G)** Adjacent non-dysplastic (AHM0040) PDO sizes measured at 196 hours and
1171 260 hours after seeding. Each point is one PDO; displacement from the diagonal reflects growth
1172 between time points. **(H)** Mean diameter-squared (proportional to number of cells) of single layer,
1173 single lumen organoids for non-dysplastic (AHM0040) and dysplastic (AHM2221) Barrett
1174 oesophagus organoids 4 and 6 days following seeding. **(I)** Time-lapse example of fusion in non-
1175 dysplastic (AHM0040) PDOs producing a single-layer, multi-lumen (SLML) morphology: at 196
1176 hours the two organoids are separate but by 204 hours they have fused; the fused organoid
1177 maintains an SLML morphology through 260 hours. **(J)** Example of a non-dysplastic (AHM0040)
1178 singe-layer single-lumen PDO interacting with and likely fusing with a single cell: in the fifth frame
1179 the single cell aligns with the organoid; in the sixth frame the single cell is no longer visible, and
1180 the organoid shows a thickened fluorescent region at the point of contact.
1181
1182 **Supplementary Figure 6: *In silico* modelling of PDO growth with variable polarity and**
1183 **polarity-inheritance. (A and B)** Overview of experimental quantification and *in silico* modelling
1184 of PDO morphology. **(A)** PDOs are imaged as z-stacks; the best-focused plane is outlined with an
1185 ellipse in Fiji and diameter is taken as the mean of minimum and maximum widths. PDOs are
1186 classified as single lumen, multi-lumen or solid; cell number is assumed to scale with diameter^2
1187 for hollow organoids and diameter^3 for solid PDOs **(B)** A 2D Cellular Potts model (CompuCell3D)
1188 simulates organoid cross-sections over Monte Carlo steps, illustrating growth, movement and

fusion over timepoints t0, t1, and t2. Each new PDO is assigned a polarity state according to an inherited polarity parameter, IP: IP=1 yields polarised, single-lumen, single-layer PDOs; IP >0 but <1 produces a mixture of lumen-forming and solid PDOs; and IP=0 produces non-polar, solid, non-luminal PDOs. Fusion events are favoured when the change in effective adhesion energy for increasing organoid–organoid contact is negative ($\Delta E_{fusion} < 0$). **(C)** Corresponding steady-state morphologies from the model: (i) a polarised PDO forming a single lumen and single epithelial layer (IP = 1), and (ii) a non-polar PDO developing as a solid, non-luminal structure (IP = 0). **(D)** Example of simulated PDO fusion: two separate single-lumen PDOs at an early time point (left) and the resulting fused multi-lumen PDO at a later time point (right). **(E)** Time course of a representative fusion event in the model, illustrating the approach, contact and remodelling of two solid PDOs into a single composite structure over successive Monte Carlo steps.

Supplementary Figure 7: Variation in transcriptional profiles across normal- and Barrett oesophagus-derived PDOs. (A) Comparisons of gastric and intestinal gene panel scores across the PDO cohort, calculated via gene set variation analysis on gene panels derived from the literature. **(B)** Unsupervised hierarchical clustering of PDOs using the top 1000 variable genes. Expression values underwent variance stabilising transformation followed by subtraction of the average expression for the respective gene. **(C)** Principal component analysis of Barrett oesophagus-derived PDOs alone. **(D and E)** Volcano plots displaying the results of differential expression analysis comparing (D) non-dysplastic and (E) dysplastic Barrett oesophagus-derived PDOs with normal gastric PDOs. Genes were coloured with relation to adjusted p-values less than 0.05 and absolute log2-fold change greater than 1. The 20 genes with the lowest adjusted p-values are annotated. **(F)** Functional enrichment analysis of up- and down-regulated DEGs specific to non-dysplastic PDOs. Full results are reported in **Supplementary Table 7**. **(G)** Functional enrichment analysis of up- and down-regulated DEGs specific to dysplastic PDOs. Full results are reported in **Supplementary Table 8**. **(H)** Comparisons of *HOXB5* and *HOXB7* gene expression across the PDO cohort. TPM-values were reported following log2-transformation. Gene expression was compared between non-dysplastic and dysplastic PDOs using a two-sample Wilcoxon test, adjusted for multiple testing using the Benjamini-Hochberg procedure.

Supplementary Figure 8: Assembloid co-cultures to explore clonal expansion.
(A) Schematic illustrating the assembloid culture system with fibroblasts under various in vitro conditions. **(B)** Brightfield and histopathological features of the assembloid cultures. Note the

similarity between Barrett oesophagus tissue (top right) and the corresponding assembloid (bottom right). **(C)** Flow cytometric analysis of fibroblasts and gating strategy. Proportion of $CD34^+$ S1 fibroblasts **(D)** and αSMA^+ S3 fibroblasts **(E)** within total fibroblasts in assembloids containing Barrett or gastric PDOs following treatment. **(F)** Schematic illustrating how clones capable of shaping a supportive niche and establishing a positive feedback loop are more likely to expand.

Supplementary Figure 9: Cytokine expression in Barrett oesophagus and oesophageal adenocarcinoma development.

Boxplots showing expression levels ($\log_2(1+TPM)$) of IL2, IFNG, IL4, IL5, IL6, IL10 and IL13 across normal gastric tissue, Barrett oesophagus (BO), and oesophageal adenocarcinoma (OAC). The data was derived from RNA-seq data from our previous study[51]. Note the significant increase in IL10.

Supplementary Figure 10: Genomic features of OAC PDOs and their relationship to parent tissue. (A and B) Correlation between tumour purity for PDOs and their adjacent tissues. Purity between PDO-tissue pairs was calculated via a pairwise Wilxocon test. **(C)** Tumour mutational burden in PDOs and their adjacent tissues. **(D)** Differences in VAFs of shared mutations across PDO-tissue pairs, with the dotted red-line indicating mutations where $VAF_{organoid} - VAF_{tissue} = 0$ (i.e. where a mutation has equal VAF in a PDO and its adjacent tissue). **(E)** Comparison of insertion-deletion (indel) mutation burden between PDOs and their adjacent tissues. **(F)** Comparison of SNV burden between PDOs and their adjacent tissues. **(G)** Comparison of ploidy in PDOs and their adjacent tissues. **(H)** Comparison of HRD index score in PDOs and their adjacent tissues. **E-H)** Red dots indicate PDO-tissue pairs with mutational concordance less than 10% and gray dots indicate PDO-tissue pairs with mutational concordance more than 10%, as reported in **Figure 5C**. Red dotted lines indicate equivalent values (i.e. y=x). Solid blue lines and shaded grey areas indicate linear regression lines of best fit alongside 95% confidence bands. Solid navy lines indicate linear regression lines following the removal of PDO-tissue pairs with mutational concordance less than 10%. Comparisons were made using Pearson correlation coefficients with R- and p- values shown. Values shown in black in the top left were calculated across all PDO-tissue pairs, and values shown in navy in the bottom right were calculated following the removal of PDO-tissue pairs with mutational concordance less than 10%. **(I)** Summary of mutational signature analysis on adjacent OAC PDOs and their parent tissues. Samples (rows) are ordered into matched PDO-tissue pairs. **(J)** Correlations in SBS17a (left) and SBS17b (right) contributions

across PDO-tissue pairs with Pearson correlation coefficients calculated separately for SBS17a and SBS17b. Annotations are the same as in (E to H). **(K)** Contributions of mutational signatures across both the gastric/BO and OAC PDO cohorts. **(L and M)** Gene set enrichment analysis of the loadings for (L) PC1 and (M) PC2 obtained from principal component analysis of RNA-seq data from the OAC PDO cohort. Analysis was based on the top 1000 variable genes across the cohort.

Supplementary Figure 11: Circos plots demonstrating genomic similarities between each OAC PDO and its parent tissue. Each plot demonstrates overall genomic landscape of the organoid (O) and adjacent tumour tissue (T). The outer circle represents the labelled chromosomes with labelled driver gene positions. The middle circle depicts copy number profile where orange shows gains and blue shows deletions. The inner most circle shows inter and intra chromosomal structural variations with grey, red, blue and green lines representing translocations, deletions, inversions and duplications, respectfully.

Supplementary Figure 12: Immunohistochemistry and fluorescence *in situ* hybridisation of *HER2*. IHC and FISH analysis of suspected HER2amplification in PDOs and matched tissue. IHC scores are shown for each tissue image based on current HER2 OAC diagnostic guidelines (0 negative, 1-2+ equivocal, 3+ positive). FISH probes and colours are indicated in the bottom left of each FISH image with chromosome 17 (CEN17q) probe shown as green and the HER2probe as red. Positive HER2 amplification determined by FISH is denoted as HER2+ in each image. All organoids and their derivation tissue are grouped together, and case IDs are labelled on the right.

Supplementary Figure 13: Morphological analysis of OAC PDOs. (A) Representative bright-field images illustrating the three morphology classes visible in an OAC PDO (CAM277): single layer, single lumen; multiple layer, single lumen; solid. Image taken at day 4. Scale bar shows 100µm. **(B)** Coexistence of multiple morphologies at day 7 of a single CAM277 culture. Scale bar shows 100µm. **(C)** Size distribution of single cells identified in a single CAM277 OAC PDO culture. Histogram shows counts per 1µm interval; dashed vertical line marks mean cell diameter. PDOs were counted as a diameter of 21µm or greater. **(D)** Size of OAC PDO (CAM277) single cells compared with Barrett oesophagus non-dysplastic (AHM0040) and dysplastic (AHM2221) PDOs. Statistical comparisons made using the Kruskal-Wallis test. **(E)** Distribution of CAM277 PDO size at days 4, 6 and 7 after seeding. **(F)** Correlation between growth at 72 hours and growth at 140 hours for 76 individual CAM277 organoids. **(G)** Representative brightfield image demonstrating

fusion of two solid CAM277 organoids between 72 and 140 hours of seeding (images taken at four hourly intervals from top left through to bottom right).

Supplementary Figure 14: Decision framework for classifying precancerous organoids. Decision tree showing how patient-derived organoids are classified by integrating patient disease grade, matching tissue, *TP53* status, mutation burden, and genomic instability. The framework emphasizes tissue-anchored, multi-modal interpretation rather than single-feature classification, and mainly to distinguish normal gastric and precancerous organoids in BO.

1298 **LIST OF TABLES**

Features / dimensions	Normal gastric organoids	Non-dysplastic BO organoids	Dysplastic BO organoids	OAC organoids
Driver gene mutations	No enrichment for OAC-selected driver events; absence of TP53 and CDKN2A alterations typical of Barrett progression	Frequent *CDKN2A* or ARID1A alterations; TP53 generally absent or subclonal	*TP53* alterations common but could be absent; *CDKN2A* or *ARID1A* frequently co-altered, or progression-associated events	High frequency of *TP53* mutations; recurrent copy number alterations affecting *CDKN2A, ARID1A, GATA6, SMAD4* and others
Genome-wide features	Very low mutation burden; no evidence of chromosomal instability or whole-genome doubling	Intermediate mutation burden (usually > 2/Mb); mutational signatures concordant with Barrett tissue even when SNV overlap is low	Higher mutation burden (usually > 4/Mb); increased genomic instability	Copy-number alterations dominate; high chromosomal instability and frequent whole-genome doubling
Transcriptome and lineage state	Strong gastric lineage program (e.g. gastric mucins, digestive enzyme genes); minimal intestinal signature. Separated from BO organoids in PCA analysis	Mixed gastric–intestinal state; inverse relationship between gastric and intestinal programs; Separated from gastric organoids in PCA analysis	Separated from gastric organoids in PCA analysis; increased expression of *HOX* genes relative to non-dysplastic BO	Diverse tumour transcriptional states; no single uniform OAC program
Morphology and growth kinetics	Benign glandular morphology; used as baseline rather than growth comparison	Four morphologies: single-layer single-lumen; single-layer multi-lumen; multi-layer luminal; solid. Luminal types expand more robustly	Loss of single-layer multi-lumen morphology; increased proportion of solid organoids	Predominantly solid morphology; cell fusion events contribute to growth variability

1299

1300 **Table 1: Integrated framework for classifying Barrett oesophagus and oesophageal**
1301 **adenocarcinoma organoids.** A decision-based framework to distinguish normal gastric and
1302 precancerous organoids are also described in **Supplementary Figure 14.**

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1306 **LIST OF SUPPLEMENTARY TABLES**

Patient ID	Age	Sex	Ethnicity	Highest grade recorded	IM present	Adjacent to OAC	Clinical trajectory after derivation
AHM0040	69	M	White British	NDBO	Y	N	Non-progressor
AHM0931	73	M	White British	NDBO	Y	N	Non-progressor
AHM1345	71	F	White British	NDBO	Y	N	Non-progressor
AHM1369	80	M	White British	NDBO	Y	N	Non-progressor
AHM1678	79	M	White British	NDBO	Y	N	Non-progressor
AHM1977	68	F	White British	NDBO	Y	N	Non-progressor
AHM2172	73	F	White British	NDBO	Y	N	Non-progressor
AHM2575	79	F	White British	NDBO	N	N	Non-progressor
AHN0070	83	M	White British	NDBO	Y	N	Non-progressor
AHM1373	74	M	White British	NDBO	Y	N	Non-progressor
AHM2149	67	M	White British	NDBO	Y	N	Non-progressor
CAM673	66	M	White British	NDBO	Y	N	No recurrence
CAM478	61	M	White British	LGD	Y	Y	No recurrence
CAM478	61	M	White British	HGD	N	Y	No recurrence
CAM603	74	M	White British	HGD	Y	Y	Non-progressor
AHM2165	77	M	White British	HGD	N	Y	No recurrence
AHM2221	74	F	White British	HGD	N	Y	Non-progressor
AHM2392	87	F	White British	HGD	-	N	Progressor
AHM2688	73	M	White British	NDBO	-	N	Non-progressor

1307

1308 **Supplementary Table 1: Demographic and clinicopathological characteristics of donors for**
1309 **PDOs obtained from non-dysplastic and dysplastic Barrett oesophagus.** - missing data. F,
1310 female; HGD, high grade dysplasia; IM, intestinal metaplasia; M, male; LGD, low grade dysplasia;
1311 NDBO, non-dysplastic Barrett oesophagus; OAC, oesophageal adenocarcinoma.

Individual ID	Age	Sex	Organoid derived from	Condition/Procedure
DE04	63	Male	GOJ	Gastrointestinal disease-free organ donor
DE26	61	Female	GOJ	Gastrointestinal disease-free organ donor
DE26	61	Female	Gastric body	Gastrointestinal disease-free organ donor
DE27	35	Male	GOJ	Gastrointestinal disease-free organ donor
DE27	35	Male	Gastric body	Gastrointestinal disease-free organ donor
DE28	37	Male	GOJ	Gastrointestinal disease-free organ donor
DE28	37	Male	Gastric body	Gastrointestinal disease-free organ donor
MS050	63	Male	Jejunum	Gastrectomy
HGH046	67	Female	Jejunum	Gastrectomy
TB16.0980	42	Male	Duodenum	Pancreaticoduodenectomy

Supplementary Table 2: A summary of demographic and clinicopathological characteristics of donors of control intestinal and gastric organoids. GOJ: gastroesophageal junction.

Day	Measure	Single layer, single lumen	Single layer, multi-lumen	Multi-layer, with lumen	Solid, multiple cells
4	Number of organoids (%)	769 (53%)	343 (23%)	139 (9%)	213 (15%)
	Max diameter (µm)	378	380	160	84.0
	Mean diameter (µm)	85.2	94.7	46.3	27.1
	Mean circularity	1.11	1.12	1.08	1.06
6	Number of organoids (%)	720 (58%)	326 (26%)	68 (6%)	119 (10%)
	Max diameter (µm)	513	397	213	83.3
	Mean diameter (µm)	106	121	63.1	27.0
	Mean circularity	1.12	1.15	1.08	1.06
7	Number of organoids (%)	776 (61%)	322 (25%)	60 (5%)	117 (9%)
	Max diameter (µm)	376	421	143	104
	Mean diameter (µm)	108	129	64.3	27.3
	Mean circularity	1.13	1.15	1.10	1.07

Supplementary Table 3: Morphological characteristics of a non-dysplastic Barrett oesophagus PDO (AHM0040) at four, six and seven days following seeding.

Day	Measure	Single layer, single lumen	Multi-layer, with lumen	Solid, multiple cells
4	Number of organoids	153 (29%)	142 (27%)	245 (45%)
	Max diameter (µm)	202	91.9	135
	Mean diameter (µm)	72.8	52.2	43
	Mean circularity	1.08	1.07	1.07
6	# organoids (%)	241 (34%)	212 (30%)	255 (36%)
	Max diameter (µm)	444	250	187
	Mean diameter (µm)	93.5	64.2	52.7
	Mean circularity	1.12	1.08	1.07

Supplementary Table 4: Morphological characteristics of a dysplastic Barrett oesophagus PDO (AHM2221) at four, six and seven days following seeding.

	Single layer, single lumen	Single layer, multi-lumen	Multi-layer, with lumen	Solid
***In vitro* observations (>1% organoids)**	BO lines only (AHM0040, AHM2221)	NBDO only (AHM0040)	BO lines only (AHM0040, AHM2221)	BO and OAC (AHM0040, AHM2221, CAM277)
***In Silico* observations**	Single organoids	Fused SLSL organoids	Single organoids	Single and fused organoids
Parameters	IP = 1 EP = True	As for SLSL	IP<1, IP>0 EP = True	IP 0 or EP False
Parameter interpretation	Cells all polar	Cells all polar	Some cells with disrupted polarity	Cells all with disrupted polarity

Supplementary Table 5: Summary of *in silico* and *in vitro* observations for the growth patterns and morphology of non-dysplastic (AHM0040) and dysplastic (AHM2221) Barrett oesophagus PDOs. BO, Barrett oesophagus; EP, establish polarity (i.e. whether cells are polar); IP, inherit polarity (i.e. the likelihood of a cell inheriting polarity); NBDO, non-dysplastic Barrett oesophagus; OAC, oesophageal adenocarcinoma; SLSL, single layer, single lumen.

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Supplementary Table 6: a summary of enriched processes and pathways relating to genes that are differentially expressed only non-dysplastic and dysplastic Barrett oesophagus versus gastric patient derived organoids. BP: biological process; GO: gene ontology; MF: molecular function; TF: transcription factor; WP: Wiki pathway.

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Supplementary table 7: a summary of enriched processes and pathways relating to genes that are differentially expressed only in non-dysplastic Barrett oesophagus versus gastric patient derived organoids. BP: biological process; GO: gene ontology; MF: molecular function; TF: transcription factor; WP: Wiki pathway.

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Supplementary table 8: a summary of enriched processes and pathways relating to genes that are differentially expressed only in dysplastic versus gastric patient derived organoids. BP: biological process; GO: gene ontology; MF: molecular function; TF: transcription factor; WP: Wiki pathway.

Solid organoids	Day 4	Day 6	Day 7
Number of organoids	1936	2013	1918
Mean diameter (µm)	43.2	47.9	49.2
Maximum diameter (µm)	183	262	213
Mean circularity	1.07	1.07	1.08
Proportion of solid organoids	99%	99%	99%

Supplementary Table 9: Morphological characteristics of an OAC PDO (CAM277) at four, six and seven days following seeding.

	5FU+ Leucovorin			Oxaliplatin			Docetaxel		
	IC50	**AUC**	**Emax**	**IC50**	**AUC**	**Emax**	**IC50**	**AUC**	**Emax**
CAM574	15.66	194.73	93.05	20.19	250.85	91.64	1.68	154.30	77.71
CAM277	8.31	191.57	93.94	7.73	169.87	95.82	2.10	177.37	78.18
CAM408	51.33	287.0	95.65	60.71	253.4^	62.89^	3.04	161.75	72.79
CAM117	14.65	210.25	88.51	96.78	246.25	76.87	2.57	140.35	93.53
CAM146	25.83	222.4	86.6	92.66*	257.0*	74.15*	1.64	214.60	50.04

* n=1 biological replicate (three technical replicates)

^ AUC and Emax were normalized to a top concentration of 100 μM. Only one biological replicate (three technical replicates) was dosed to 200 μM

Supplementary Table 10: OAC PDO sensitivity to cytotoxic chemotherapy. AUC: area under the curve (an integral value of viability following exposure to defined concentrations of each drug); E_{max}: the maximum effect; IC_{50}: the concentration of each drug required to reduce viability to 50%; 5FU: 5-fluorouracil.

ID	Source	Age	Sex	Surgical TNM	Chemotherapy regimen	Histopathological grading	TRG score
CAM473	Resection	78	M	T3N0Mx	ECX	Poor	3
CAM621	Resection	57	M	T1bN1M0	ECX	Poor	3
CAM453	Staging Lap	69	M	T3N1M0	ECX	Poor	4
CAM453	Resection	69	M	T3N1M0	ECX	Poor	4
CAM296	Resection	72	M	T3N3Mx	ECF	Poor	4
CAM486	Resection	72	M	T4aN0M0	ECX	Moderate	4
CAM540	Resection	52	M	T3N0M0	ECX	Poor	4
CAM535	Resection	72	F	T3N1M0	ECX	Poor	4
CAM522	Resection	62	M	T4aN1M0	ECX	Poor	4
CAM247	Resection	67	M	T2N2M0	ECX	Moderate	4
CAM292	Resection	66	F	T3N1Mx	ECX	Poor	4
CAM388	Resection	78	M	T2N0M0	ECX	Poor	4
CAM429	Resection	77	M	T2N0M0	ECX	Moderate	4
CAM479	Resection	63	M	T3N0M0	ECX	Poor	4
CAM433	Resection	66	M	T3N2M0	ECX	Poor	4
CAM629	Resection	61	M	T3N1M0	FLOT	Moderate	4
CAM664	Resection	62	M	T3N0M0	FLOT	Moderate	4
CAM665	Resection	61	F	T3N3M0	FLOT	Poor	4
CAM668	Resection	57	M	T3N3M0	FLOT	Poor	4
CAM676	Resection	59	M	T3N1M0	FLOT	Poor	4
CAM401	Resection	77	F	T4aN2M0	CF	Poor	5
CAM467	Resection	68	M	T2N0M0	ECX	Poor	5
CAM542	Resection	77	M	T3N2M0	ECX	Poor	5
CAM338	Resection	51	F	T3N1Mx	ECX	Poor	5
CAM423	Resection	55	M	T3N2M0	ECX	Poor	5
CAM446	Resection	68	F	T4aN3M0	ECX	Poor	5
CAM576	Resection	51	M	T4bN1	FLOT	Poor	5
CAM614	Resection	76	M	T4aN3M0	FLOT	Moderate	5
CAM667	Resection	62	M	T3N3M0	FLOT	Moderate	5
CAM421	Staging Lap	57	M	-	CF	Poor	Not available*
CAM408	Resection	60	M	T1aN0M0	None	Moderate	n/a
CAM412	Resection	51	F	T1bN0M0	None	Poor	n/a
CAM450	Resection	76	M	T1N0M0	None	Moderate	n/a
CAM471	Resection	82	M	T3N3M0	None	Poor	n/a
CAM277	Resection	80	F	T3N2M0	None	Poor	n/a
CAM501	Staging Lap	72	M	T3N2M0	ECX	Moderate	n/a
CAM574	Resection	71	M	T3N1M0	None	Moderate	n/a
OESO117	Unknown	61	M	T3 N2 M0	None	-	n/a
OESO146	Unknown	73	M	T2 N0 M0	None	-	n/a

Supplementary Table 11: Demographic and clinicopathological characteristics of organoids derived from patients with oesophageal adenocarcinoma. All patients were White British. *TRG not available but sample noted demonstrate therapy-related tumour regression. - missing data. ECX, epirubicin, cisplatin, capecitabine; CF, cisplatin, 5-fluorouracil; FLOT: 5-fluorouracil/leucovorin, oxaliplatin, docetaxel; Lap laparoscopy; TRG, tumour regression grade.

	CAM277	CAM401	CAM408	CAM412	CAM429	CAM501	CAM535	CAM574
0 Gy	1	1	1	1	1	1	1	1
2 Gy	0.86	0.88	1.03	0.77	0.71	0.42	0.58	0.62
4 Gy	0.80	0.62	0.99	0.56	0.66	0.54	0.57	0.78
6 Gy	0.74	0.77	1.10	0.52	0.53	0.36	0.59	0.70
8 Gy	0.68	0.75	0.73	0.62	0.60	0.24	0.47	0.55
AUC	0.83	0.79	0.98	0.66	0.67	0.46	0.63	0.70

Supplementary Table 12: OAC PDO sensitivity to x-ray irradiation. Organoid forming efficiency shown for each PDO following exposure to 0-8Gy x-ray irradiation in a single fraction. AUC, median area under the curve (integral of 0-8Gy organoid forming efficiency values).

Organoid	IC_{50} (geometric mean, μM)	IC_{50} (range, μM)	Observed E_{max} (mean, %)	Observed E_{max} (range, %)	AUC
CAM277	0.78	0.57–0.95	71.99	62.14–85.26	314.87
CAM401	4.17	3.17–5.48	38.80	31.46–46.14	368.55
CAM429	0.57	0.37–0.89	49.14	36.27–62.02	317.40
CAM535	0.8	0.61–1.04	60.37	46.74–74.00	318.35

Supplementary Table 13: OAC PDO sensitivity to the CDK4/6 inhibitor abemaciclib. AUC: area under the curve (an integral value of viability following exposure to defined concentrations of each drug); E_{max}: the maximum effect; IC_{50}: the concentration of each drug required to reduce viability to 50%.

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Supplementary Table 14: Metadata relating to archived data for models generated in this study.

Figure 1

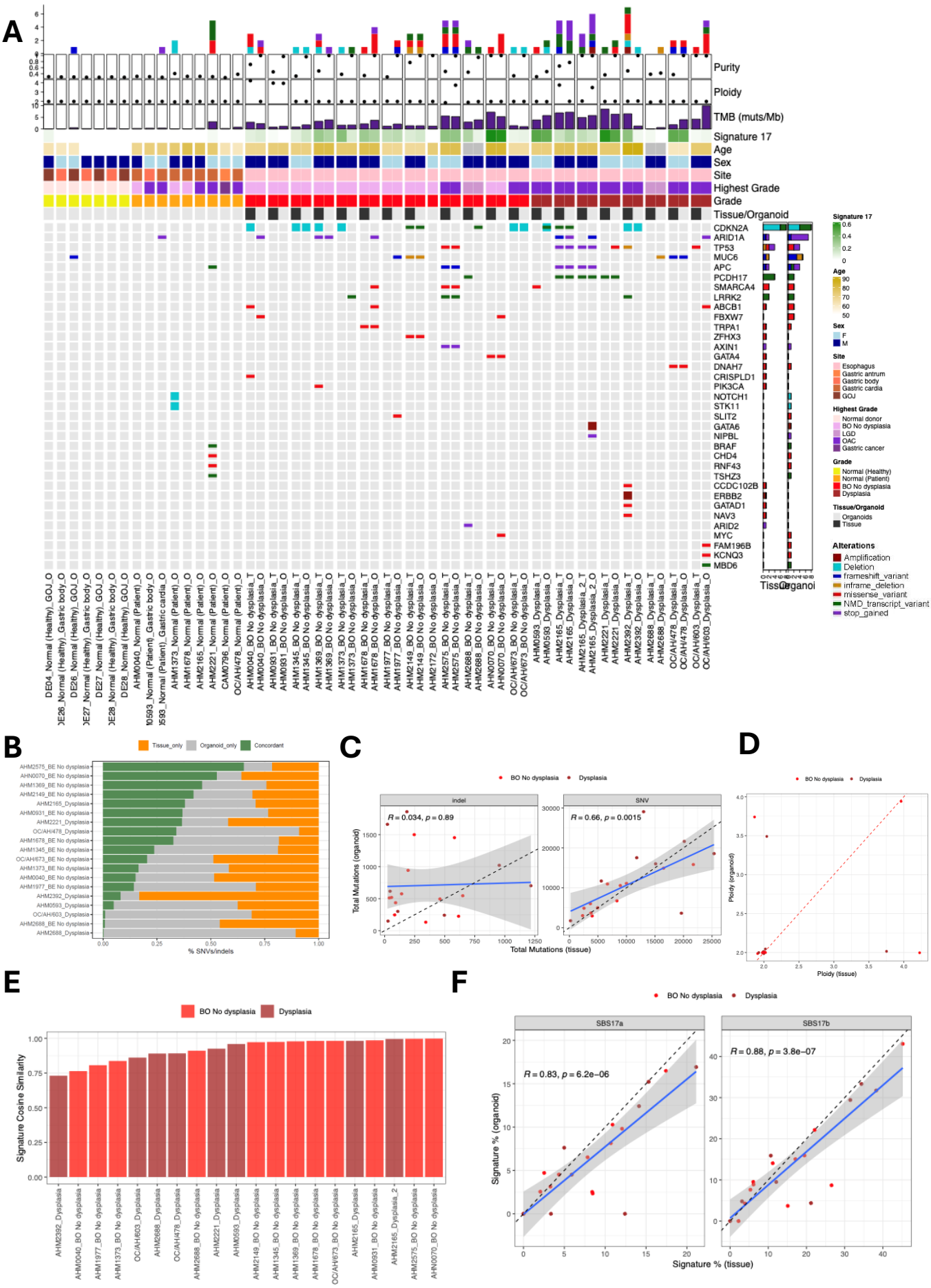

A
Purity
Ploidy
TMB (muts/Mb)
Signature 17
Age
Sex
Site
Highest Grade
Grade
Tissue/Organoid
CDKN2A
ARID1A
TP53
MUC6
APC
PCDH17
SMARCA4
LRRK2
ABCB1
FBXW7
TRPA1
ZFHX3
AXIN1
GATA4
DNAH7
CRISPLD1
PIK3CA
NOTCH1
STK11
SLIT2
GATA6
NIPBL
BRAF
CHD4
RNF43
TSHZ3
CCDC102B
ERBB2
GATAD1
NAV3
ARID2
MYC
FAM196B
KCNQ3
MBD6
Tissue
Organoid
Signature 17
Age
Sex
F
M
Site
Esophagus
Gastric antrum
Gastric body
Gastric cardia
GOJ
Highest Grade
Normal donor
BO No dysplasia
LGD
OAC
Gastric cancer
Grade
Normal (Healthy)
Normal (Patient)
BO No dysplasia
Dysplasia
Tissue/Organoid
Organoids
Tissue
Alterations
Amplification
Deletion
frameshift_variant
inframe_deletion
missense_variant
NMD_transcript_variant
stop_gained
B
Tissue_only
Organoid_only
Concordant
% SNVs/indels
C
BO No dysplasia
Dysplasia
indel
SNV
R = 0.034, p = 0.89
R = 0.66, p = 0.0015
Total Mutations (organoid)
Total Mutations (tissue)
D
BO No dysplasia
Dysplasia
Ploidy (organoid)
Ploidy (tissue)
E
BO No dysplasia
Dysplasia
Signature Cosine Similarity
F
BO No dysplasia
Dysplasia
SBS17a
SBS17b
R = 0.83, p = 6.2e−06
R = 0.88, p = 3.8e−07
Signature % (organoid)
Signature % (tissue)

Figure 2

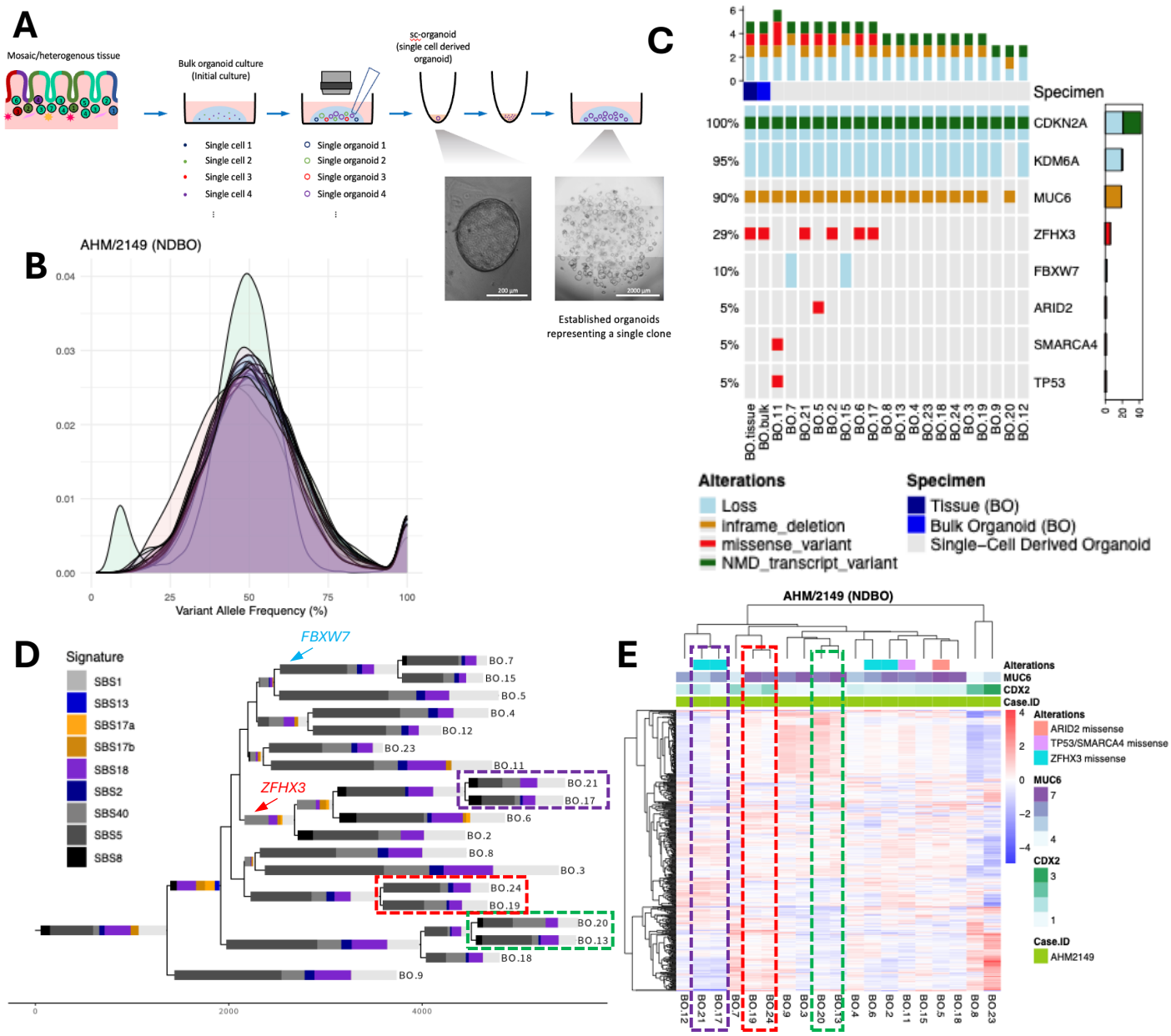

A
Mosaic/heterogenous tissue
Bulk organoid culture
(Initial culture)
sc-organoid
(single cell derived
organoid)
Single cell 1
Single cell 2
Single cell 3
Single cell 4
Single organoid 1
Single organoid 2
Single organoid 3
Single organoid 4
Established organoids
representing a single clone
B
AHM/2149 (NDBO)
Variant Allele Frequency (%)
C
Specimen
CDKN2A
KDM6A
MUC6
ZFHX3
FBXW7
ARID2
SMARCA4
TP53
Alterations
Loss
inframe_deletion
missense_variant
NMD_transcript_variant
Specimen
Tissue (BO)
Bulk Organoid (BO)
Single-Cell Derived Organoid
D
Signature
SBS1
SBS13
SBS17a
SBS17b
SBS18
SBS2
SBS40
SBS5
SBS8
FBXW7
ZFHX3
E
AHM/2149 (NDBO)
Alterations
MUC6
CDX2
Case.ID
ARID2 missense
TP53/SMARCA4 missense
ZFHX3 missense
AHM2149

Figure 3

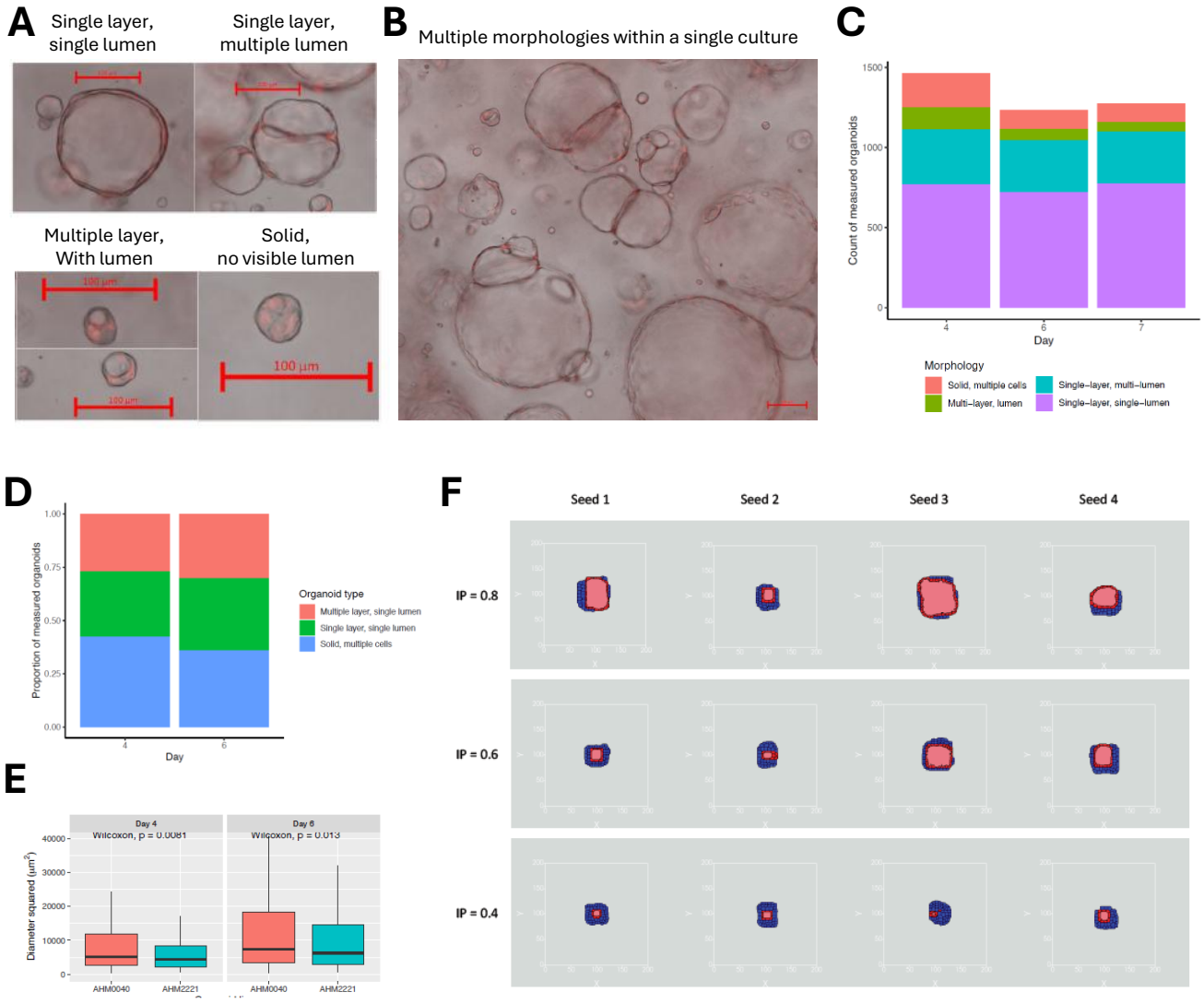

A
Single layer, single lumen
Single layer, multiple lumen
Multiple layer, With lumen
Solid, no visible lumen
100 µm
B
Multiple morphologies within a single culture
C
Count of measured organoids
Day
Morphology
Solid, multiple cells
Multi-layer, lumen
Single-layer, multi-lumen
Single-layer, single-lumen
D
Proportion of measured organoids
Day
Organoid type
Multiple layer, single lumen
Single layer, single lumen
Solid, multiple cells
E
Day 4
Wilcoxon, p = 0.0081
Day 6
Wilcoxon, p = 0.013
Diameter squared (µm²)
AHM0040
AHM2221
F
Seed 1
Seed 2
Seed 3
Seed 4
IP = 0.8
IP = 0.6
IP = 0.4

Figure 4

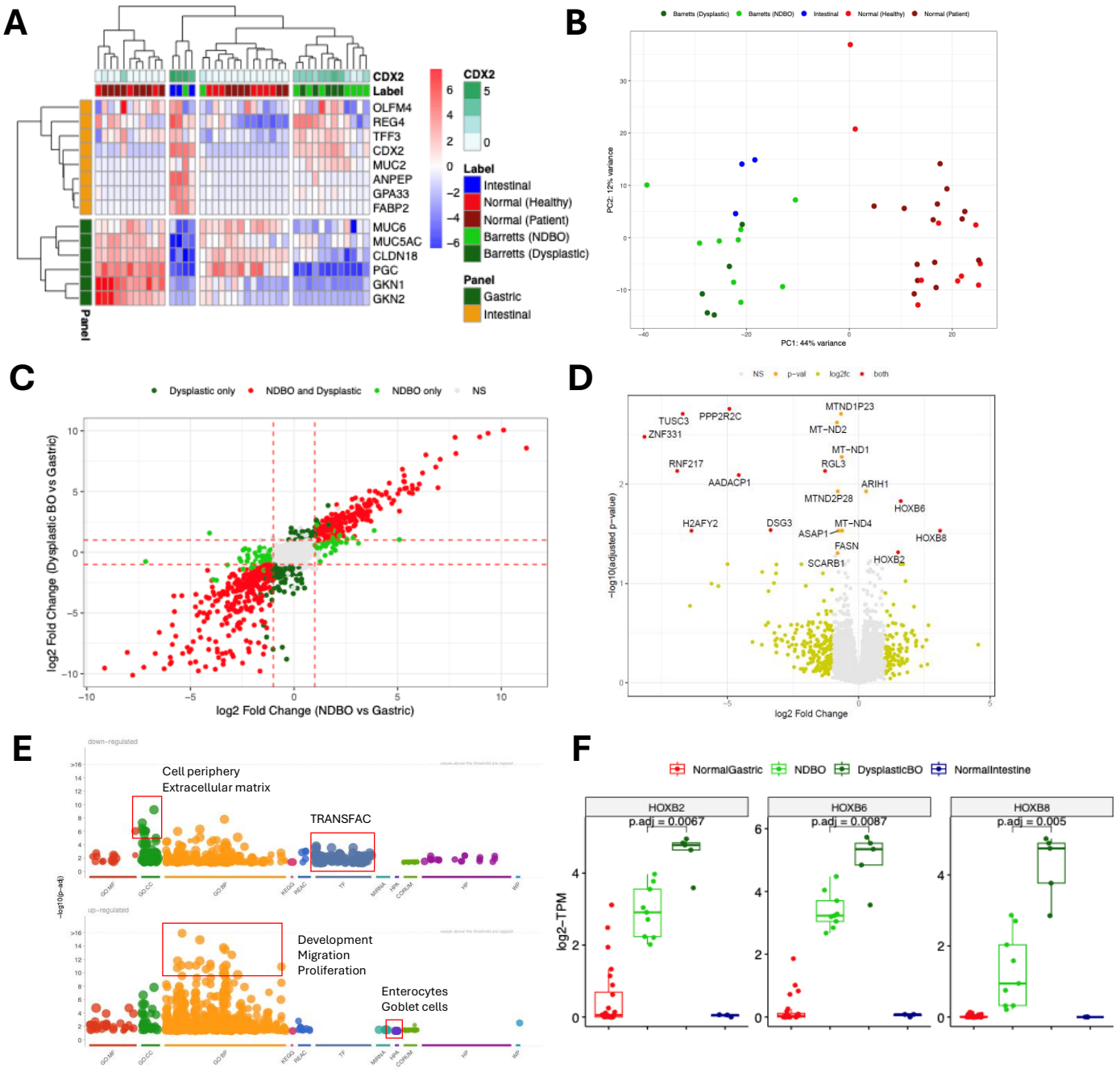
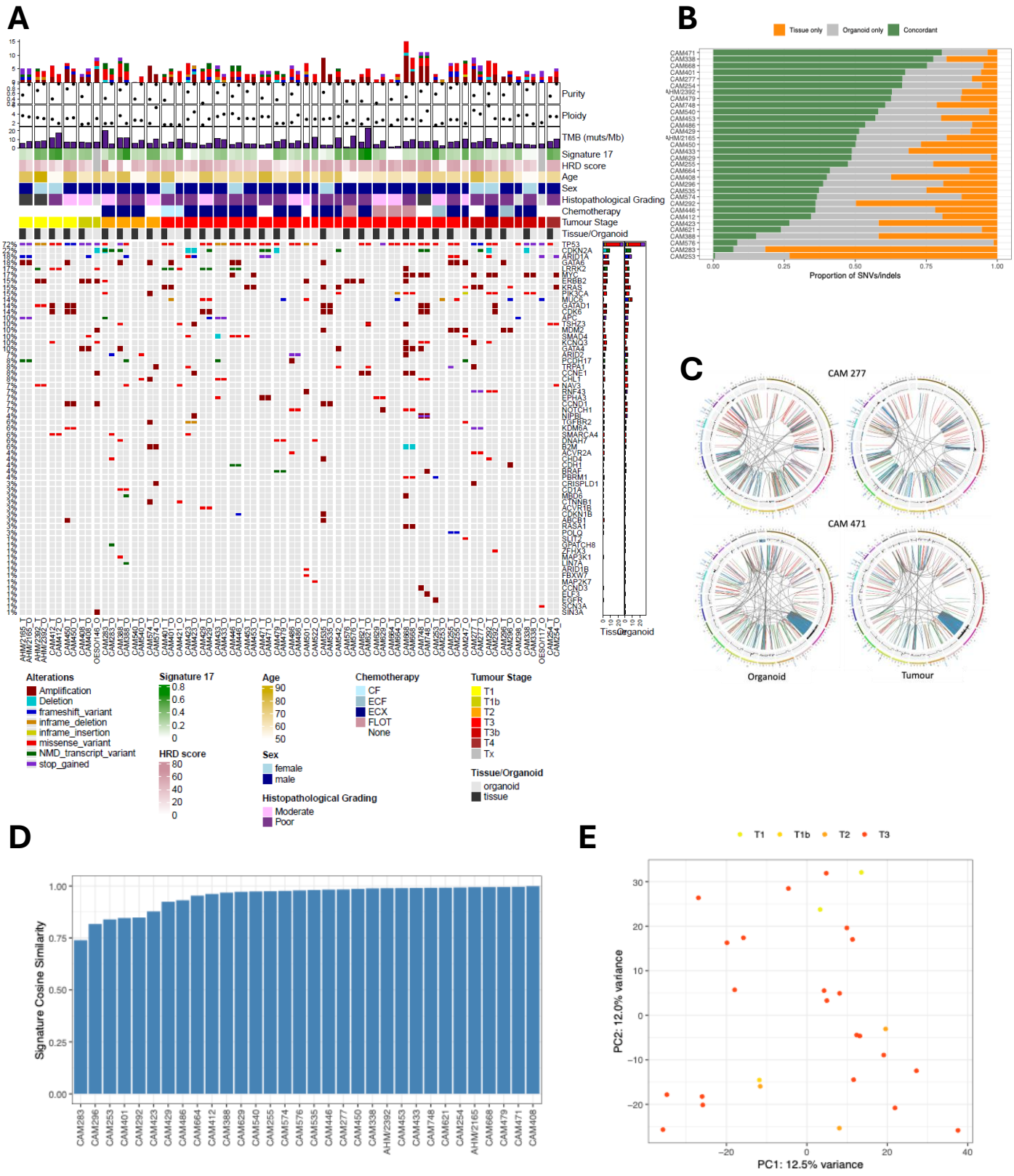

A
CDX2
Label
OLFM4
REG4
TFF3
CDX2
MUC2
ANPEP
GPA33
FABP2
MUC6
MUC5AC
CLDN18
PGC
GKN1
GKN2
Panel
Intestinal
Normal (Healthy)
Normal (Patient)
Barretts (NDBO)
Barretts (Dysplastic)
Gastric
B
Barretts (Dysplastic)
Barretts (NDBO)
Intestinal
Normal (Healthy)
Normal (Patient)
PC2: 12% variance
PC1: 44% variance
C
Dysplastic only
NDBO and Dysplastic
NDBO only
NS
log2 Fold Change (Dysplastic BO vs Gastric)
log2 Fold Change (NDBO vs Gastric)
D
NS
p-val
log2fc
both
MTND1P23
TUSC3
PPP2R2C
ZNF331
MT-ND2
MT-ND1
RNF217
RGL3
AADACP1
ARIH1
MTND2P28
HOXB6
H2AFY2
DSG3
MT-ND4
ASAP1
HOXB8
FASN
SCARB1
HOXB2
−log10(adjusted p-value)
log2 Fold Change
E
down-regulated
Cell periphery
Extracellular matrix
TRANSFAC
up-regulated
Development
Migration
Proliferation
Enterocytes
Goblet cells
−log10(p-adj)
F
NormalGastric
NDBO
DysplasticBO
NormalIntestine
HOXB2
p.adj = 0.0067
HOXB6
p.adj = 0.0087
HOXB8
p.adj = 0.005
log2−TPM

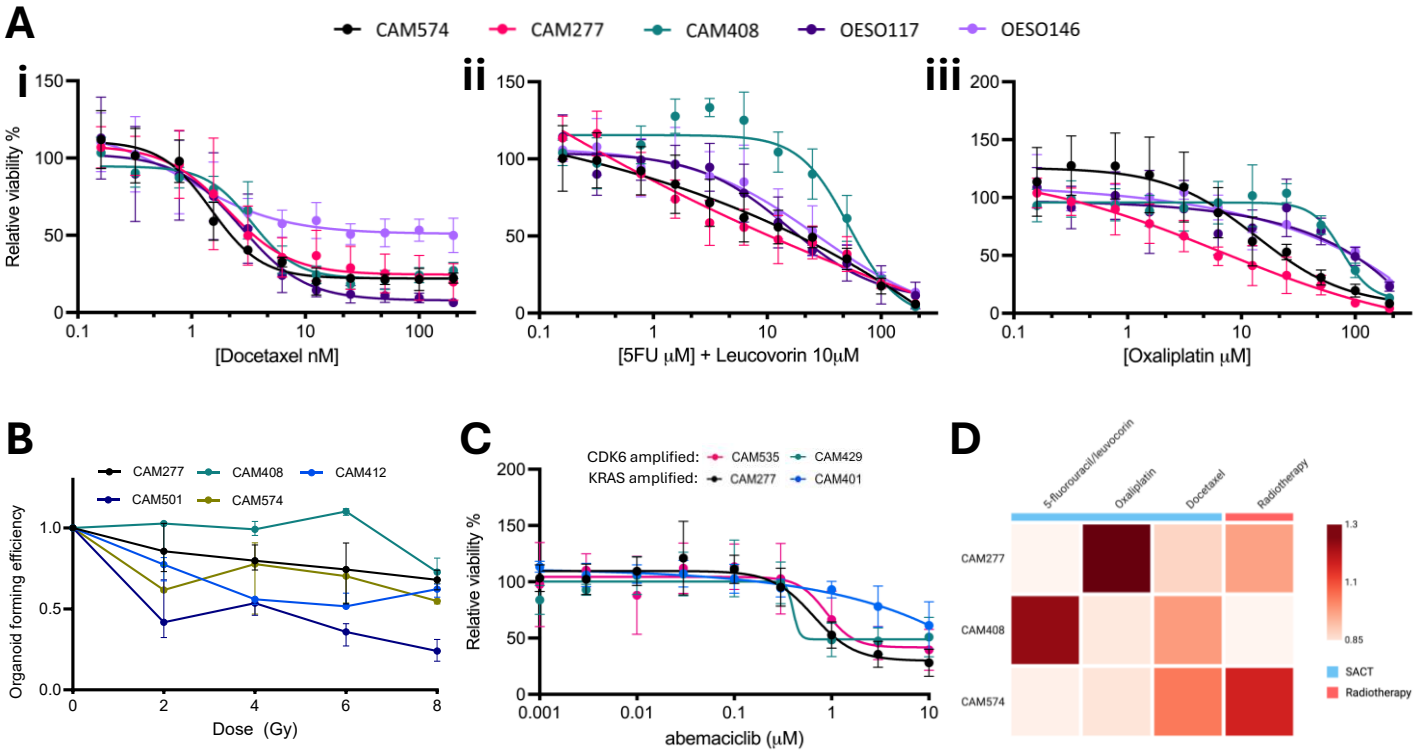
Figure 5


A
Purity
Ploidy
TMB (muts/Mb)
Signature 17
HRD score
Age
Sex
Histopathological Grading
Chemotherapy
Tumour Stage
Tissue/Organoid
Alterations
Amplification
Deletion
frameshift_variant
inframe_deletion
inframe_insertion
missense_variant
NMD_transcript_variant
stop_gained
Signature 17
HRD score
Age
Sex
female
male
Histopathological Grading
Moderate
Poor
Chemotherapy
CF
ECF
ECX
FLOT
None
Tumour Stage
T1
T1b
T2
T3
T3b
T4
Tx
Tissue/Organoid
organoid
tissue
B
Tissue only
Organoid only
Concordant
Proportion of SNVs/indels
C
CAM 277
CAM 471
Organoid
Tumour

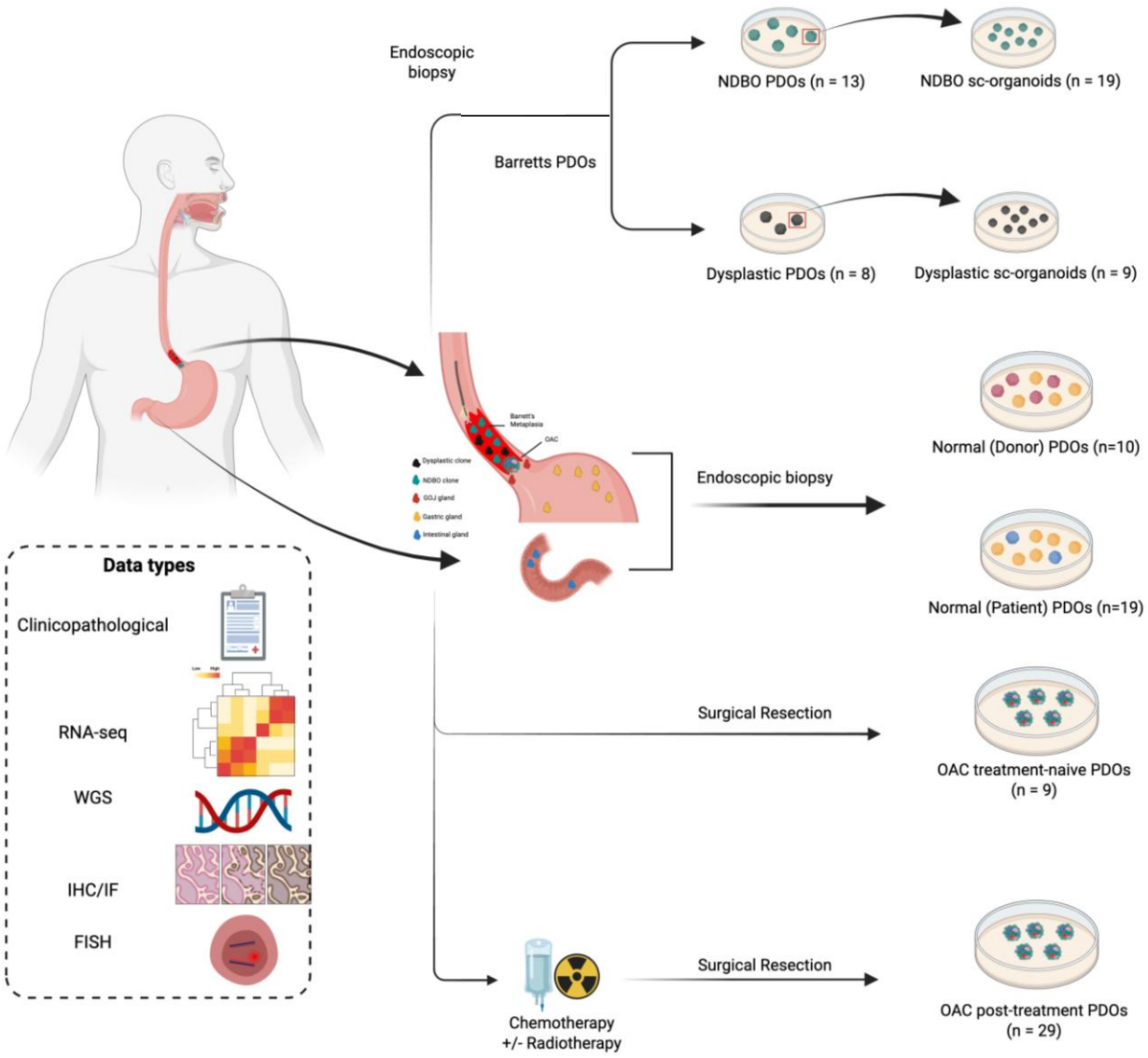
D
Signature Cosine Similarity

E
T1
T1b
T2
T3
PC2: 12.0% variance
PC1: 12.5% variance

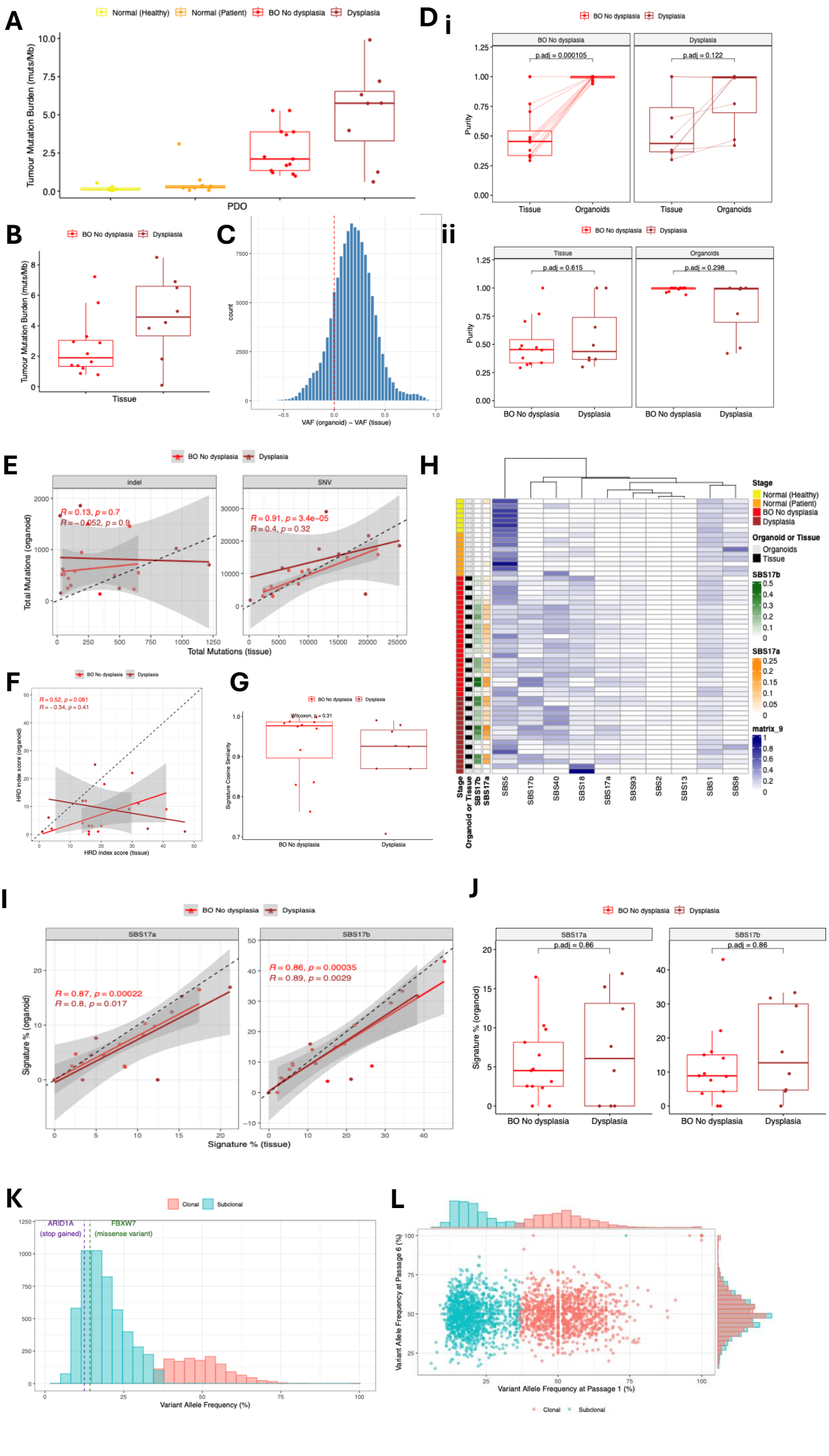
Figure 6


A
CAM574
CAM277
CAM408
OESO117
OESO146
i
Relative viability %
[Docetaxel nM]
ii
[5FU μM] + Leucovorin 10μM
iii
[Oxaliplatin μM]
B
CAM277
CAM408
CAM412
CAM501
CAM574
Organoid forming efficiency
Dose (Gy)
C
CDK6 amplified:
CAM535
CAM429
KRAS amplified:
CAM277
CAM401
Relative viability %
abemaciclib (μM)
D
5-fluorouracil/leuvocorin
Oxaliplatin
Docetaxel
Radiotherapy
CAM277
CAM408
CAM574
SACT
Radiotherapy

Supplementary Figure 1

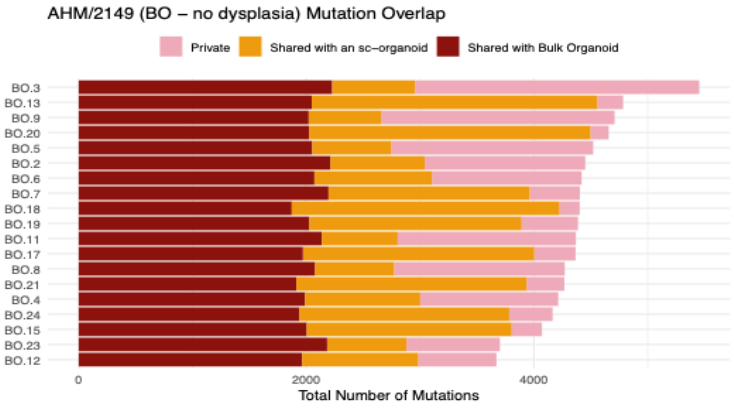


Supplementary Figure 2

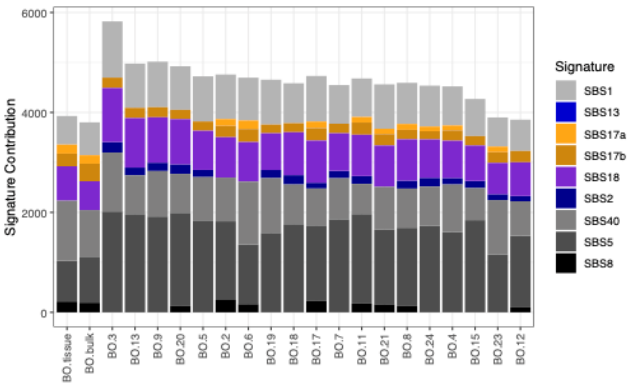


Supplementary Figure 3

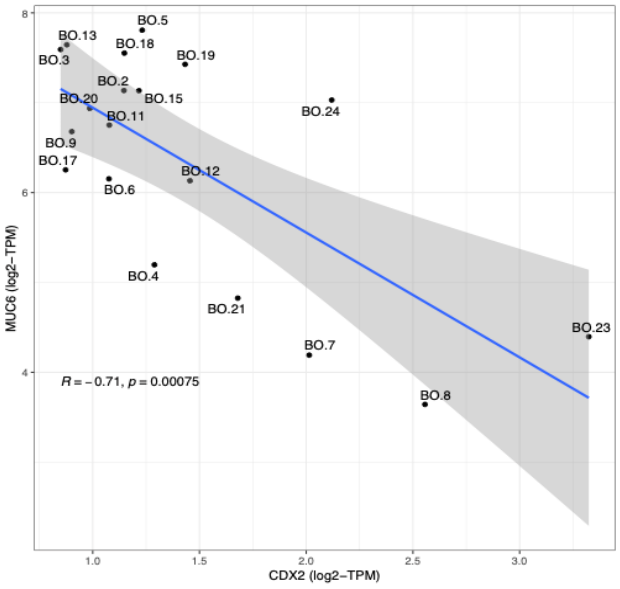
A



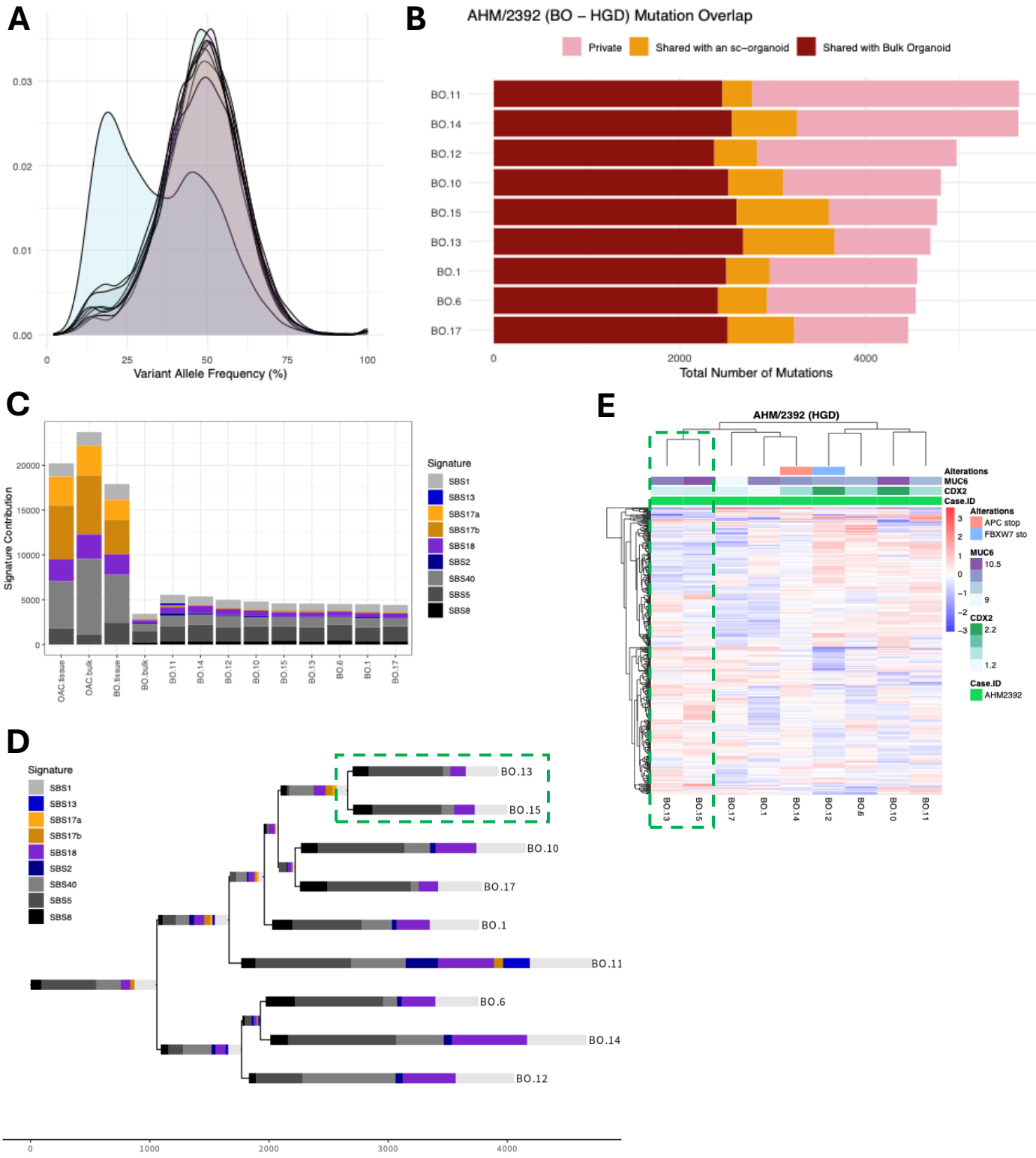
B

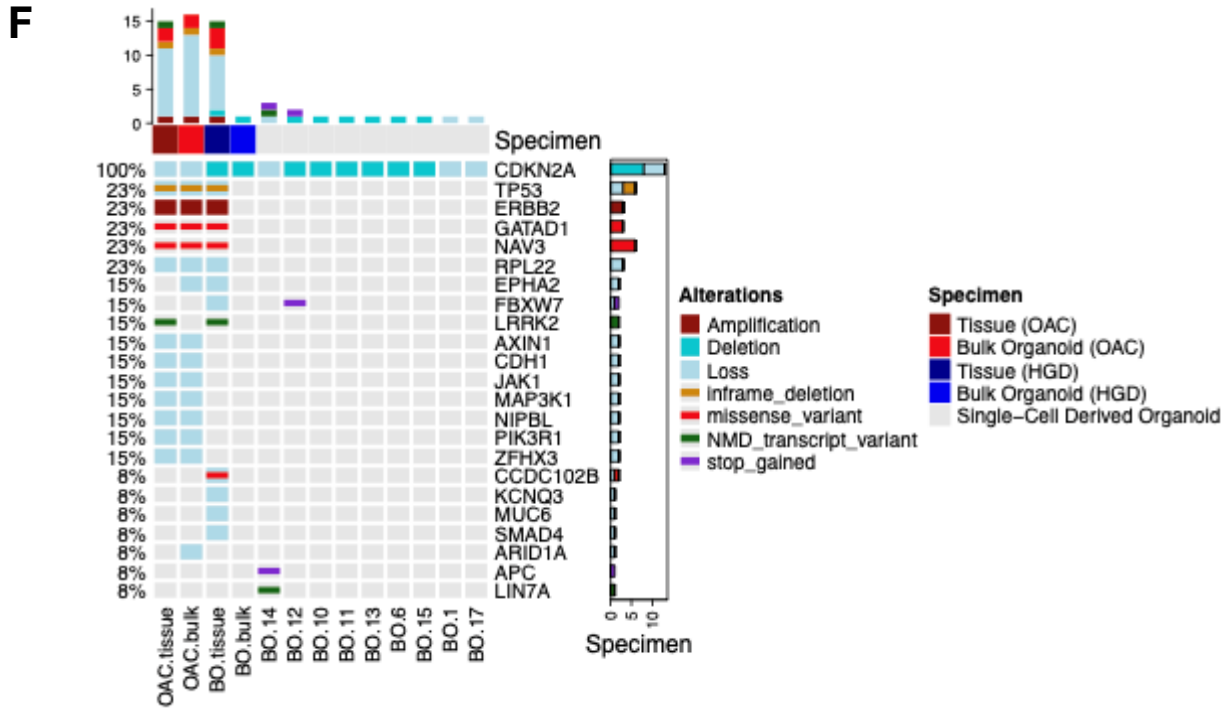


C

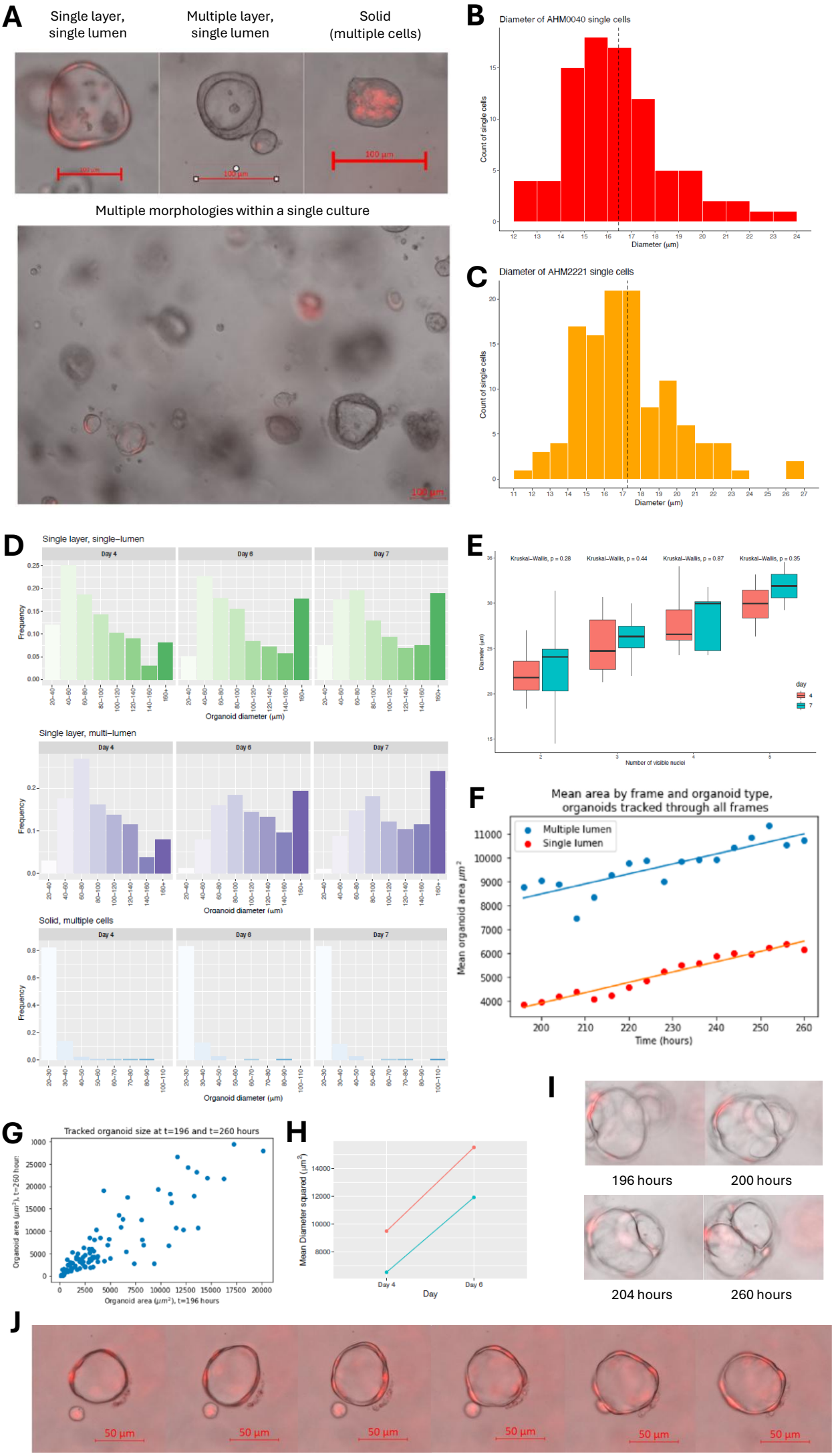


Supplementary Figure 4



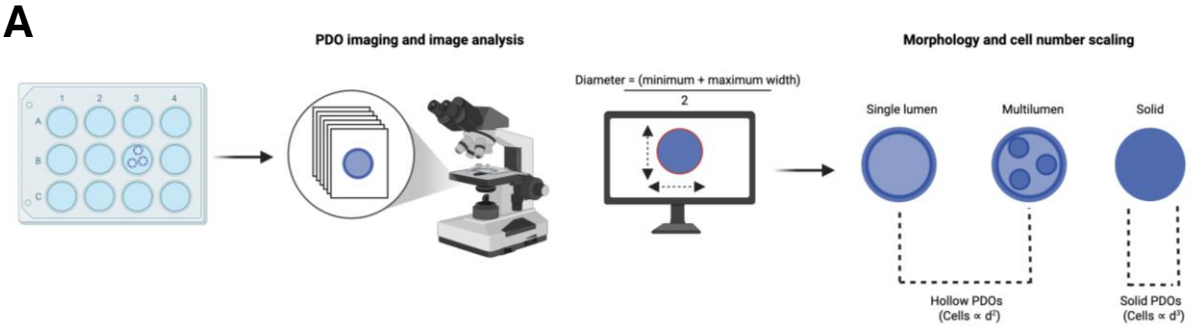


Supplementary Figure 5

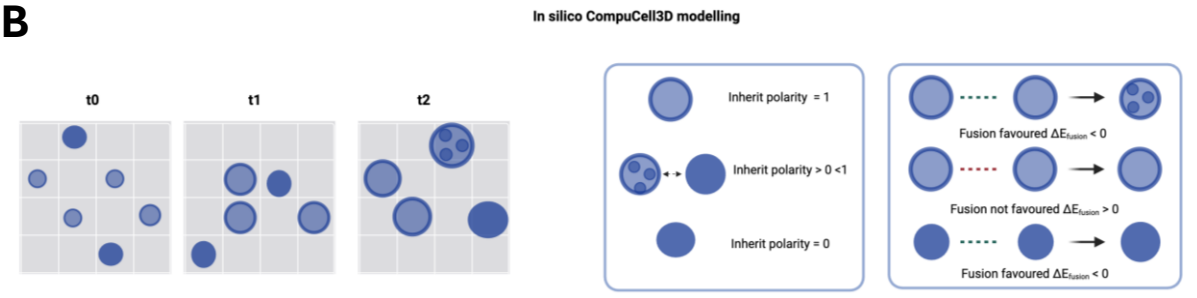


Supplementary Figure 6

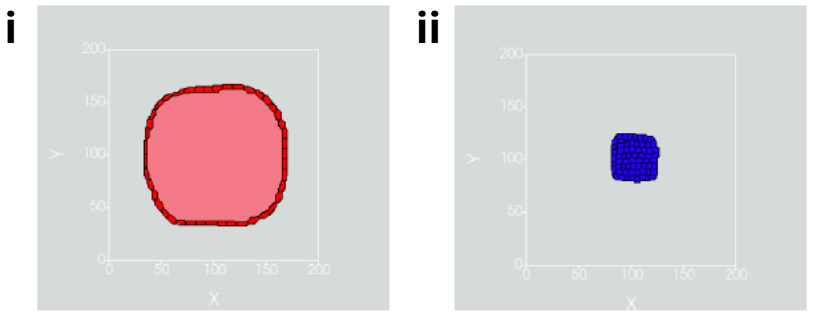
A



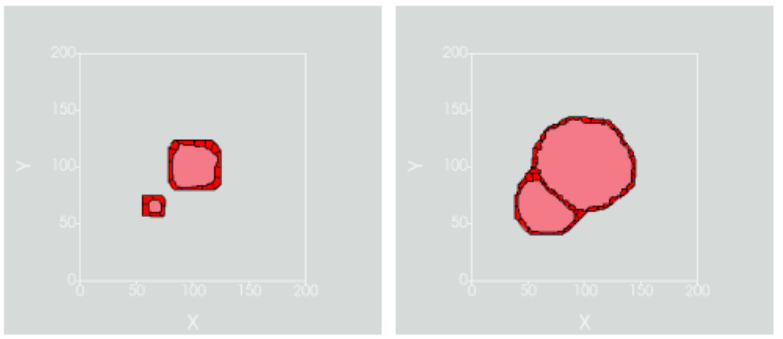
B



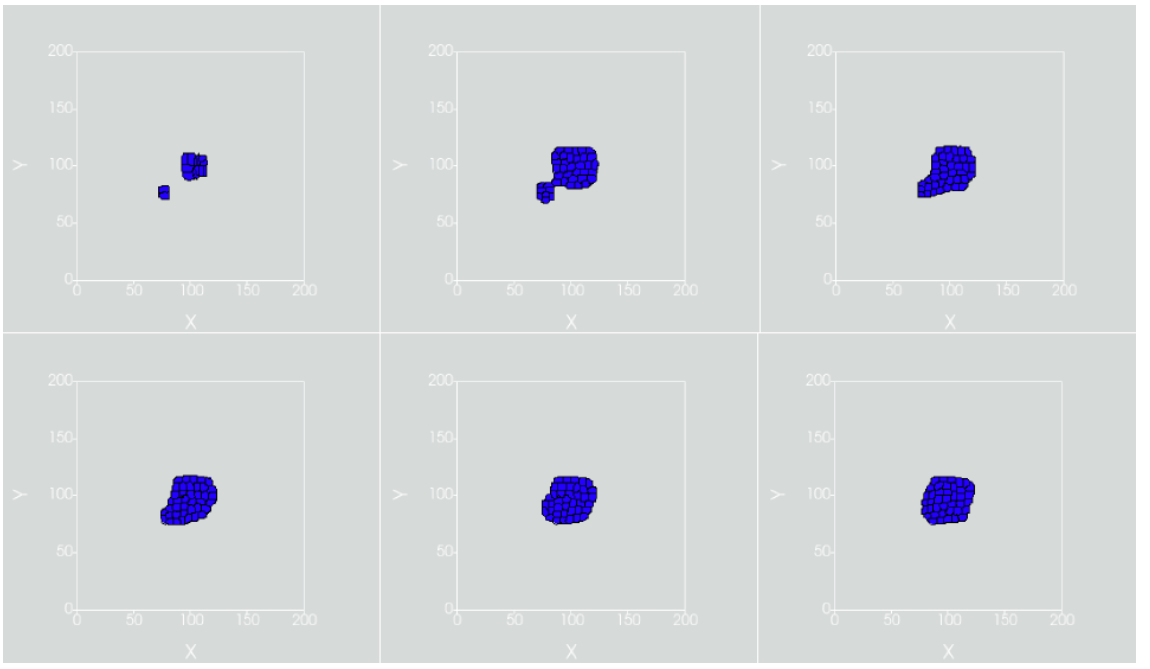
C



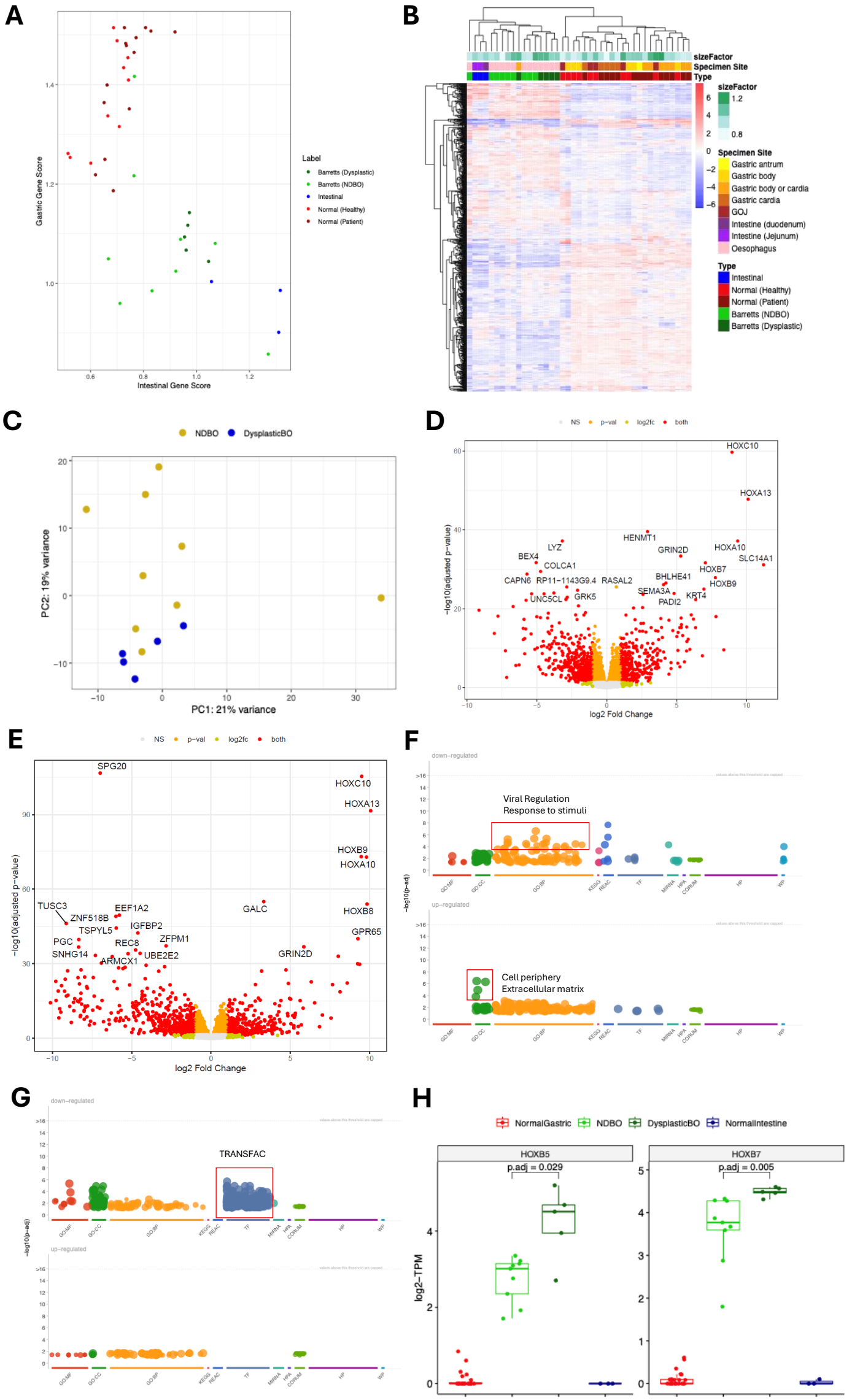
D



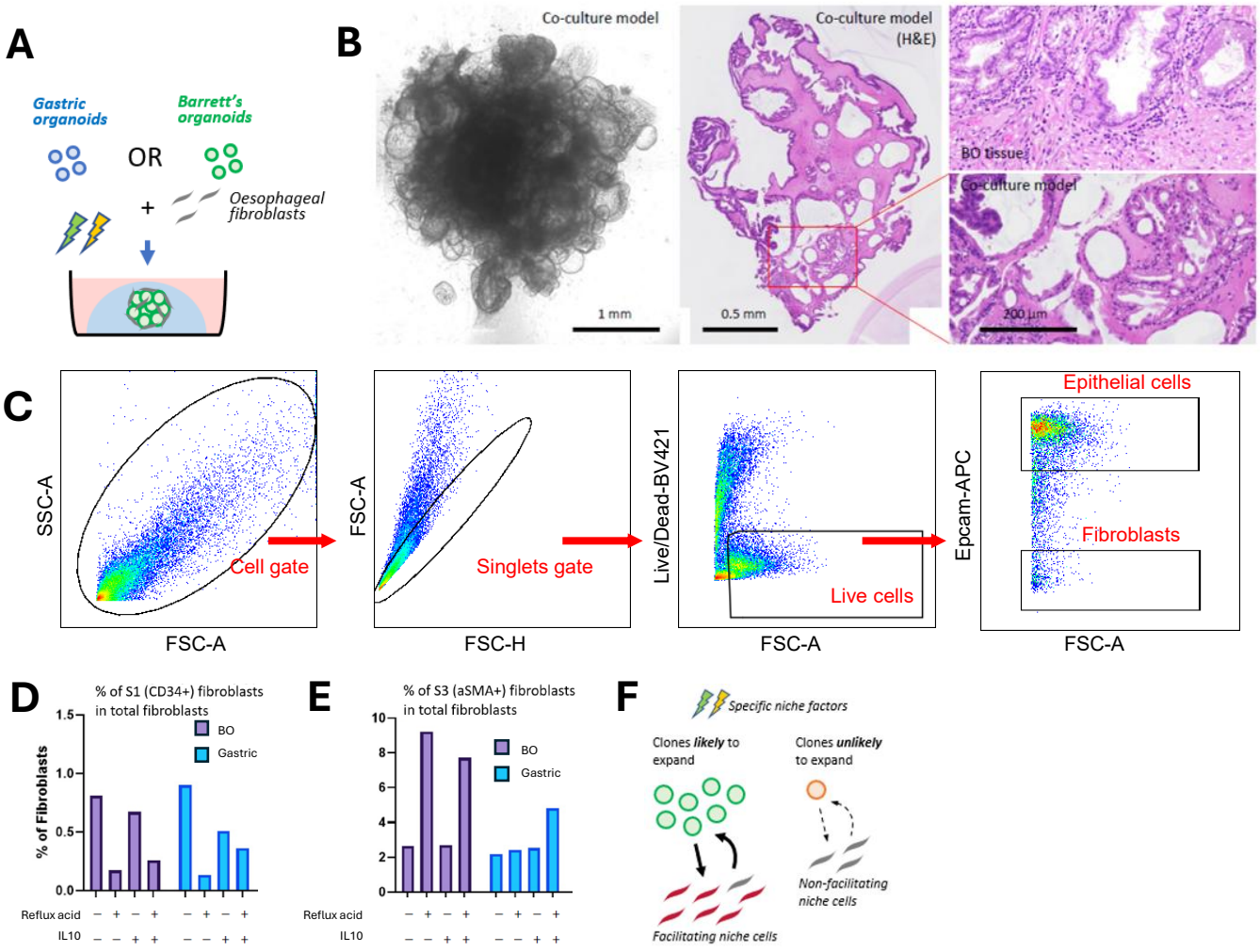
E



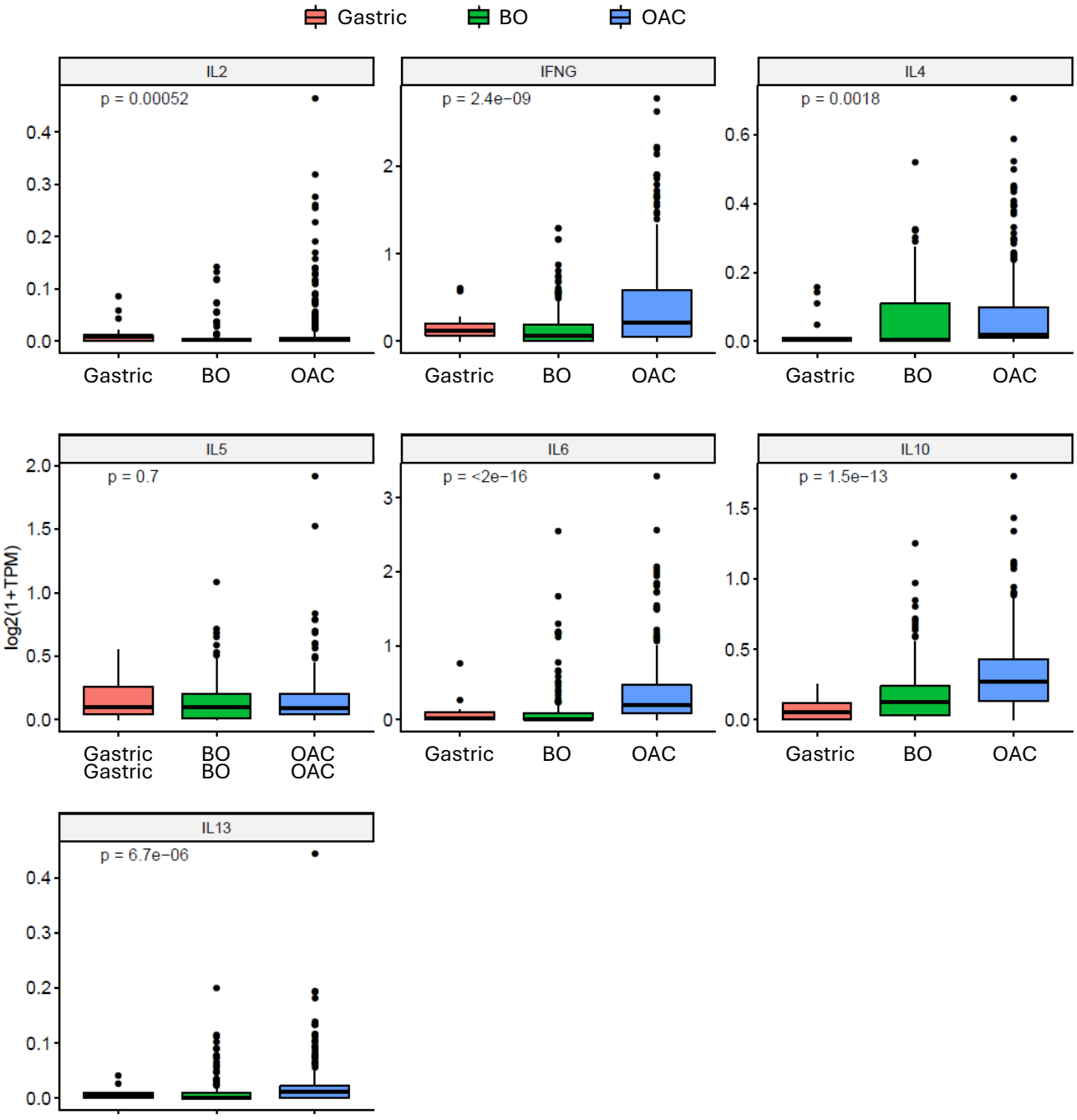
Supplementary Figure 7



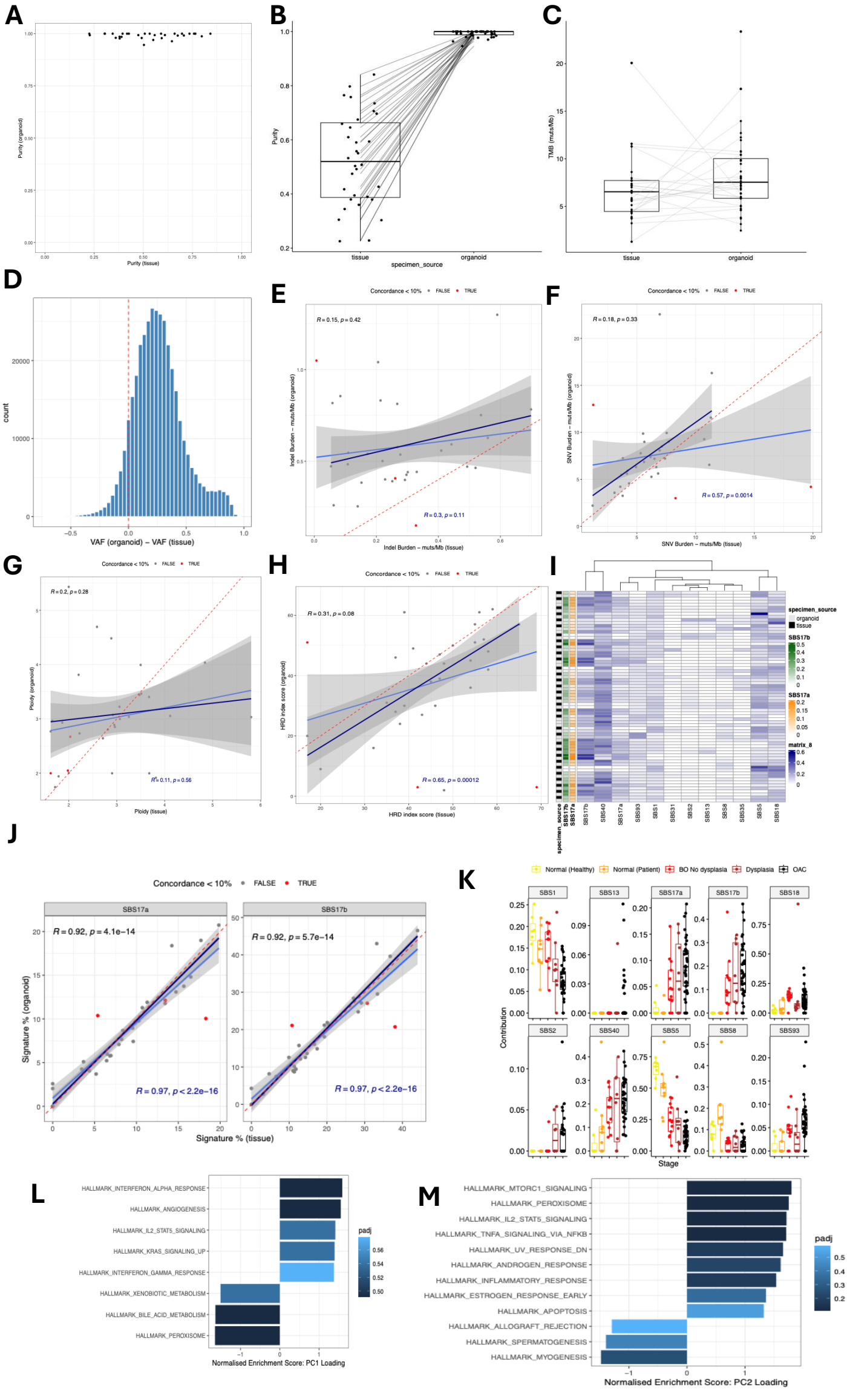
Supplementary Figure 8



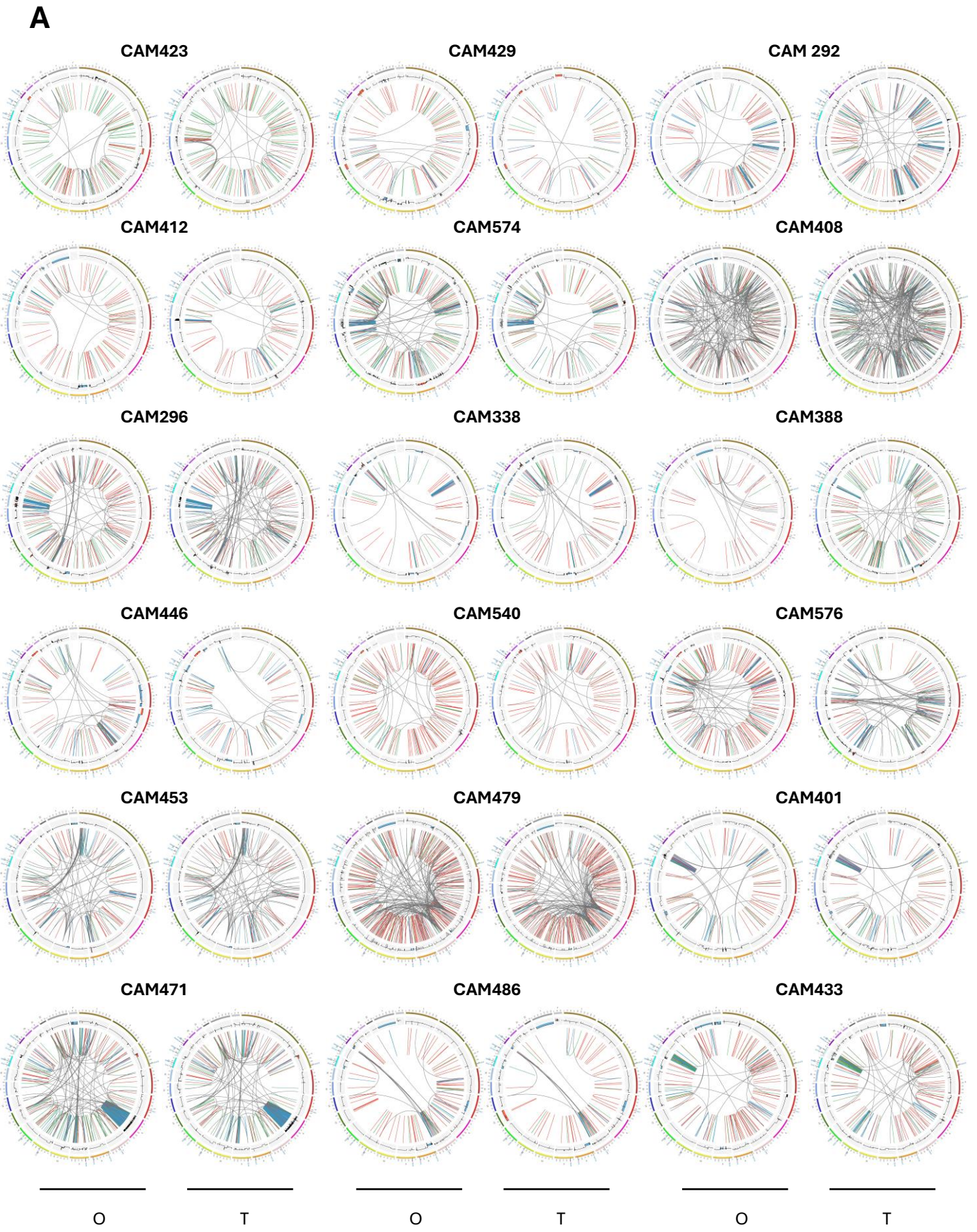
Supplementary Figure 9

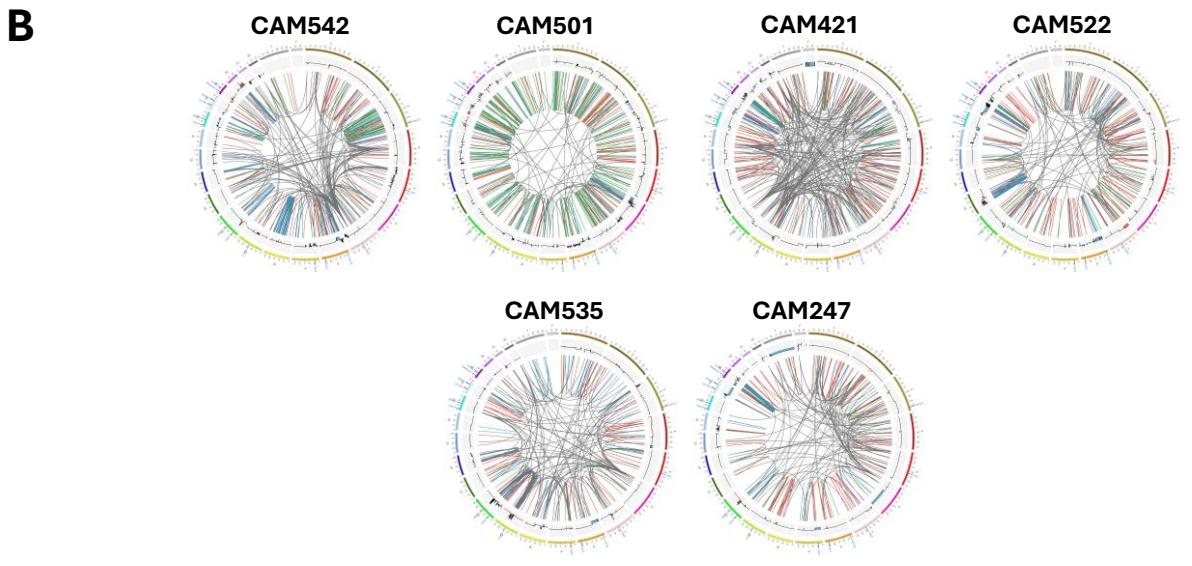


Supplementary Figure 10

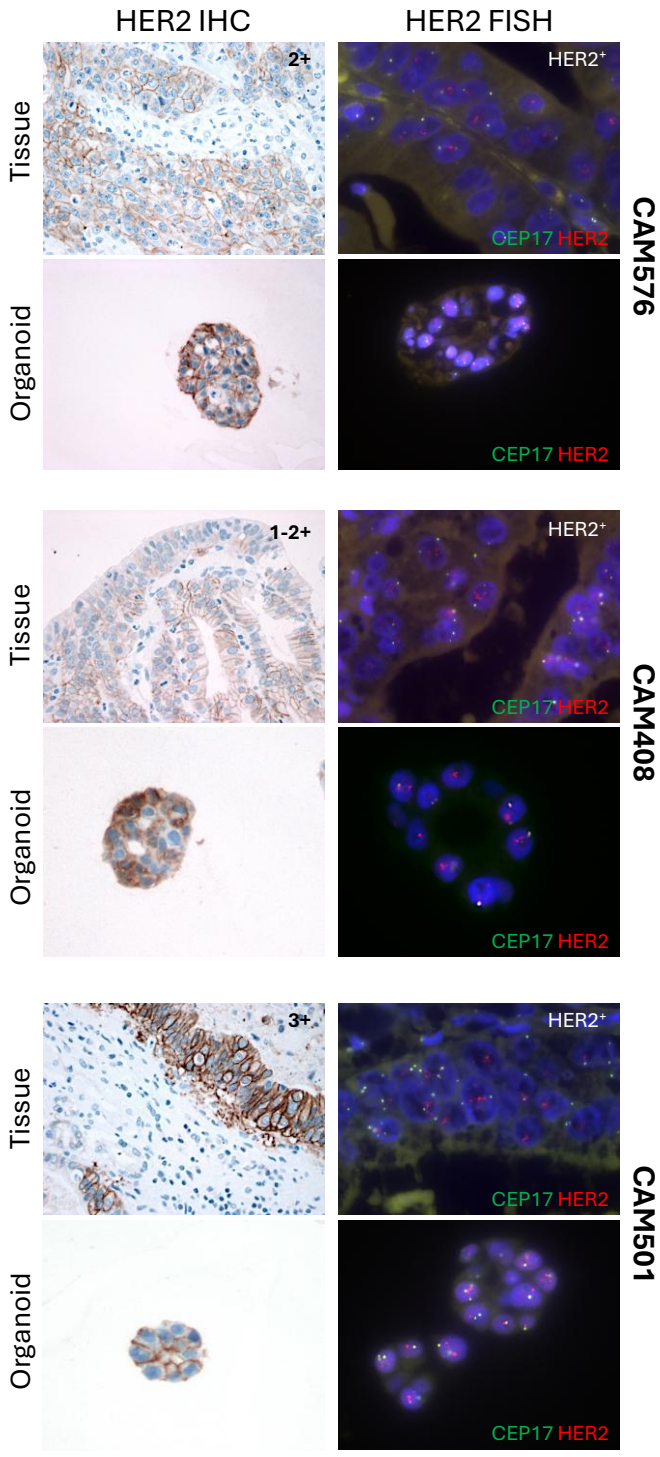


Supplementary Figure 11

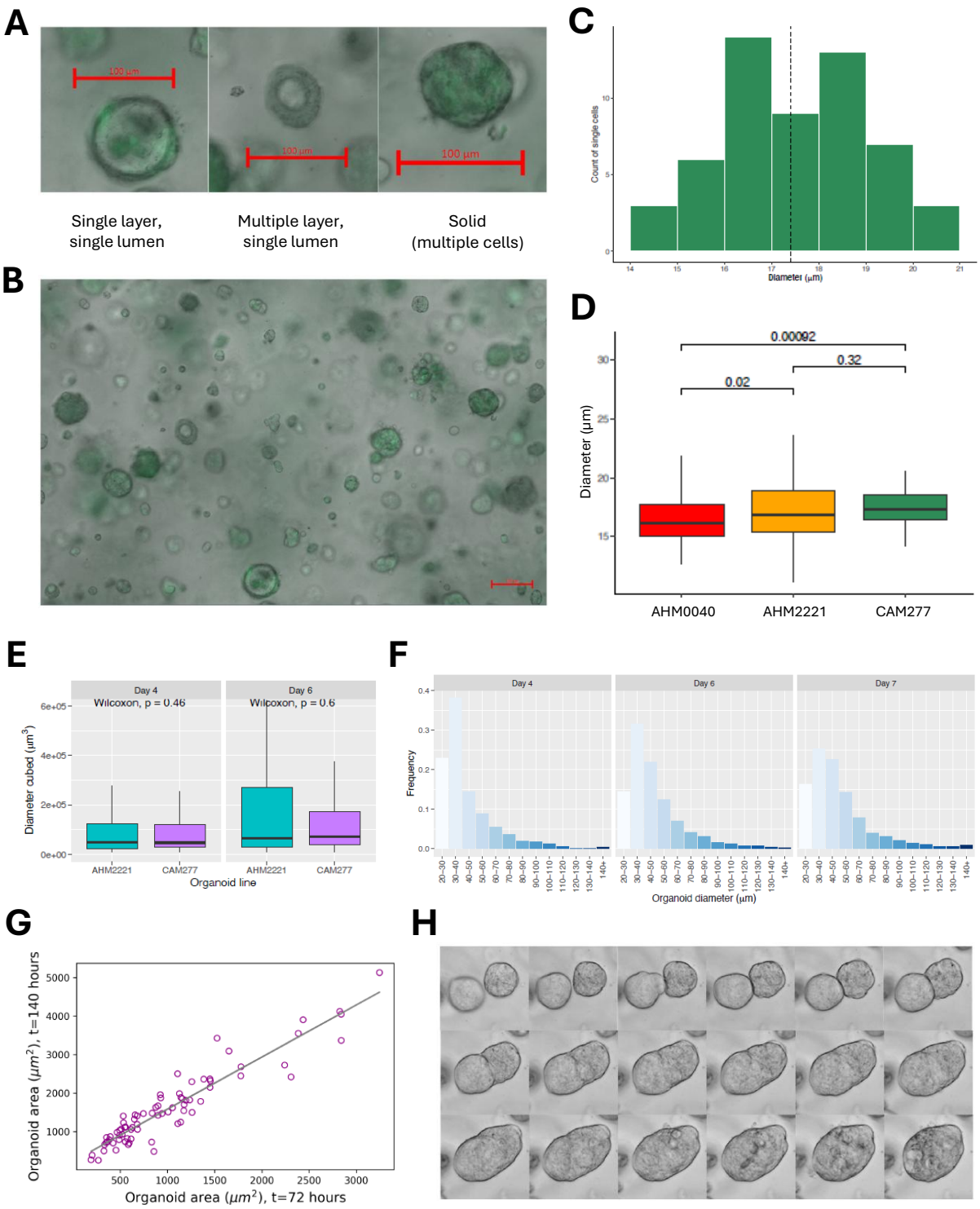
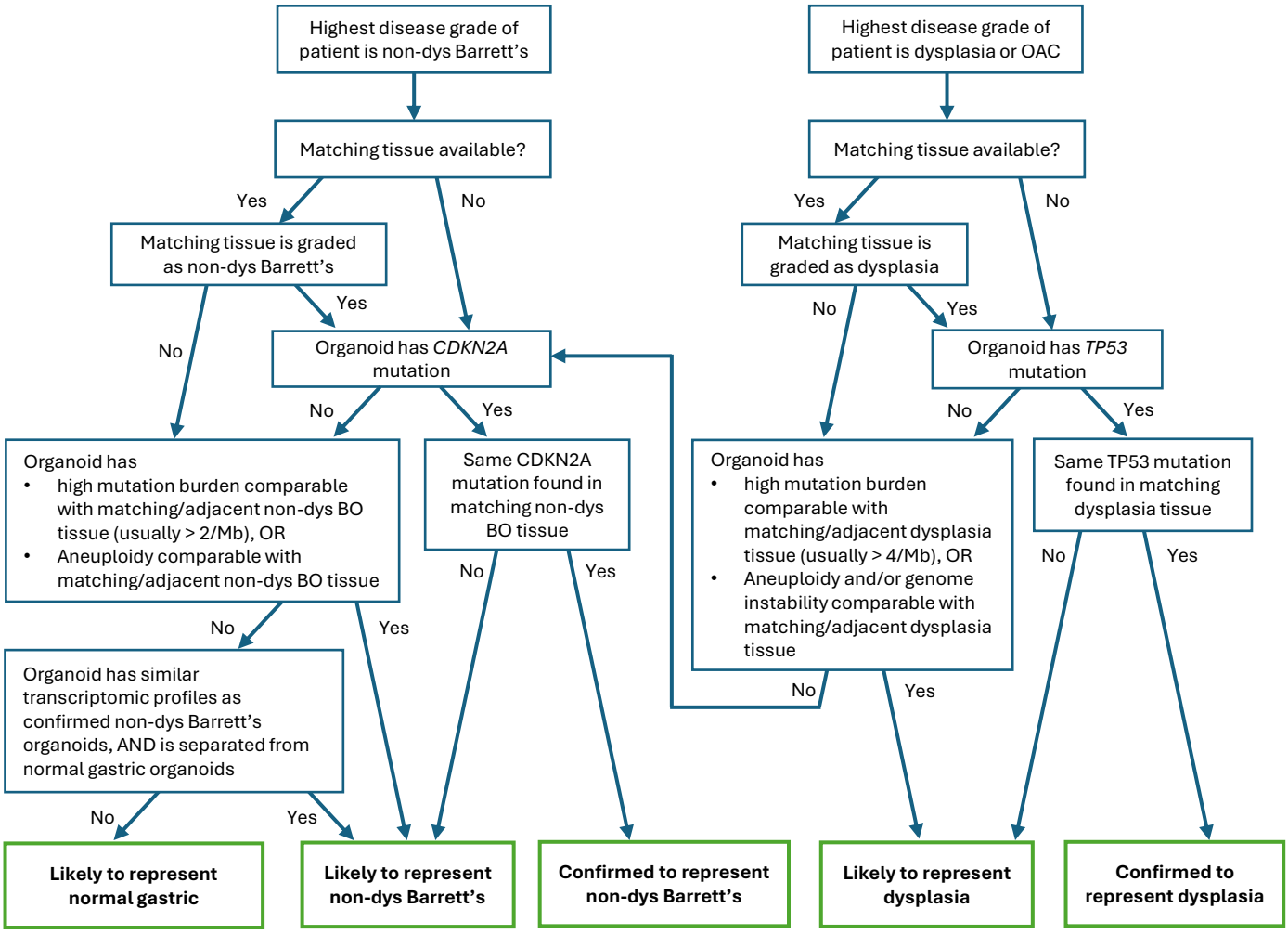




Supplementary Figure 12



Supplementary Figure 13



Supplementary Figure 14