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1 **In-depth clinical and biological exploration of DNA Damage Immune Response (DDIR) as a**
2 **biomarker for oxaliplatin use in colorectal cancer**

3
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28

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31

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36

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44 from AstraZeneca, Merckgroup and Psioxus unrelated to this work.
45

46 **Abstract**

47 **Purpose:** The DNA Damage Immune Response (DDIR) assay was developed in breast cancer
48 (BC) based on biology associated with deficiencies in homologous recombination and
49 Fanconi Anemia (HR/FA) pathways. A positive DDIR call identifies patients likely to respond
50 to platinum-based chemotherapies in breast and oesophageal cancers. In colorectal cancer
51 (CRC) there is currently no biomarker to predict response to oxaliplatin. We tested the
52 ability of the DDIR assay to predict response to oxaliplatin-based chemotherapy in CRC and
53 characterised the biology in DDIR-positive CRC.

54 **Methods:** Samples and clinical data were assessed according to DDIR status from patients
55 who received either 5FU or FOLFOX within the FOCUS trial (n=361, stage 4), or neo-adjuvant
56 FOLFOX in the FOxTROT trial (n=97, stage 2/3). Whole transcriptome, mutation and
57 immunohistochemistry data of these samples were used to interrogate the biology of DDIR
58 in CRC.

59 **Results:** Contrary to our hypothesis, DDIR negative patients displayed a trend towards
60 improved outcome for oxaliplatin-based chemotherapy compared to DDIR positive patients.
61 DDIR positivity was associated with Microsatellite Instability (MSI) and Colorectal Molecular
62 Subtype 1 (CMS1). Refinement of the DDIR signature, based on overlapping interferon-
63 related chemokine signalling associated with DDIR positivity across CRC and BC cohorts,
64 further confirmed that the DDIR assay did not have predictive value for oxaliplatin-based
65 chemotherapy in CRC.

66 **Conclusions:** DDIR positivity does not predict improved response following oxaliplatin
67 treatment in CRC. However, data presented here suggests the potential of the DDIR assay in
68 identifying immune-rich tumours that may benefit from immune checkpoint blockade,
69 beyond current use of MSI status.

70 **Introduction**

71

72 Colorectal cancer (CRC) is the fourth most common cancer and the second most common
73 cause of cancer related death in the UK (1). CRC diagnostic classification relies on the WHO
74 classification and the tumour-node-metastasis (TNM) staging system. While histological
75 assessment provides valuable prognostic information, it cannot identify specific patient
76 subgroups within tumour type, grade or clinical stage that respond best to chemotherapy.
77 Despite advances in treatment regimens, 5-year overall survival (OS) rates in the
78 unresectable metastatic setting remain at 10% (2). In patients with stage III or histologically
79 high-risk stage II tumours, recurrence is seen in 45% and 16% of patients respectively,
80 following surgery and adjuvant 5-FU based chemotherapy (2). The addition of oxaliplatin to
81 5-FU based regimens has led to a 20% risk reduction in OS following surgery for patients
82 with stage III CRC (3–5). However chronic peripheral neuropathy occurs in ~50% of patients
83 exposed to oxaliplatin (6), and there is no clinically-validated test available to predict
84 oxaliplatin response. Therefore, a significant proportion of patients may endure distressing
85 side effects from this treatment with no clinical benefit (7). This highlights the need for the
86 development of improved predictive tools to guide treatment decision making and
87 ultimately improve patient outcomes (8).

88

89 Numerous models suggest that conventional chemotherapy elicits high levels of DNA
90 damage and DNA strand breaks in highly proliferative cancer cells that can either prime
91 them for cell death, or tip already primed cells into apoptosis (9). The efficacy of
92 chemotherapy in cancer cells is often compromised due to dysfunctional damage detection
93 or cell death mechanisms, allowing cell survival (9). Certain chemotherapeutic agents target

94 vulnerabilities inherent in tumours with defective DNA damage repair machinery, leading to
95 neoplastic cell death. In CRC, the most common defective DNA damage repair mechanism
96 occurs in tumours with microsatellite instability (MSI), characterised by defects in DNA
97 mismatch repair. MSI tumours account for ~15% of stage II/III CRC and ~4% of stage IV
98 patients, and are largely characterised by hypermutation, an increase in cancer-specific
99 neoantigen production, high immune infiltration, and a favourable prognosis in earlier
100 stages (10,11). Interestingly, in the recent FOxTROT neoadjuvant colon cancer
101 chemotherapy clinical trial, this immune-rich MSI subgroup, defined by loss of MMR,
102 specifically failed to gain a clear significant benefit from oxaliplatin-based neoadjuvant
103 therapy (7). The DNA damage immune response (DDIR) signature, which comprises a 44-
104 gene transcriptional signature based on loss of the Fanconi anemia/BRCA (FA/BRCA) DNA
105 damage response pathway, was previously developed in breast cancer (BC), where it
106 demonstrated clinical utility for the identification of patients with a good response to
107 anthracycline and/or cyclophosphamide-based neoadjuvant chemotherapy (12,13). DDIR-
108 positive tumours (exhibiting defective DNA damage repair) are characterised by an
109 inflammatory tumour microenvironment (TME), upregulation of interferon signalling genes
110 and high lymphocytic infiltration. Additional studies in BC indicated that DDIR-positive
111 tumours have increased levels of CXCL10 and enhanced signalling through the cGAS/STING
112 pathway (14).

113

114 Given these predictive findings, the Stratification in COloRecTal cancer (S:CORT) consortium
115 (15) hypothesised that the DDIR signature would be predictive of oxaliplatin benefit in CRC,
116 based on its ability to predict benefit from DNA-damaging therapy in BC. In this study we
117 tested the ability of the DDIR signature to identify patients that may respond to oxaliplatin-

118 based chemotherapy in both metastatic and neoadjuvant CRC settings, employing
119 transcriptional profiling and bioinformatic analysis of subsets of samples from the FOCUS
120 (first-line metastatic, n=391) and FOxTROT (first-line neoadjuvant, n=97 randomised
121 controlled trials. We ascertained if DDIR-positivity was associated with improved outcomes
122 in metastatic CRC patients treated with FOLFOX compared to 5FUFA alone (bolus and
123 infusional 5-FU and folinic acid on the modified de Gramont schedule), and in patients with
124 localised disease treated with FOLFOX in the neo-adjuvant setting. We also performed a
125 series of analyses to comprehensively characterise the underlying biology of DDIR subtypes
126 in CRC compared to BC.

127

128 **Word Count = 633**

129

130

131

132 **Materials and Methods**

133

134 As part of the MRC Stratified Medicine in Colorectal Cancer Consortium (S:CORT) (15),
135 tumour biospecimens with associated clinical trial data were identified for exploration of
136 potential stratifiers for oxaliplatin treatment. The randomised MRC FOCUS trial was selected
137 for exploration in the metastatic setting and the FOxTROT trial was selected for exploration
138 of short course FOLFOX in the neoadjuvant setting.

139

140 ***FOCUS Trial***

141 FOCUS was a large UK-based randomised controlled trial comparing different strategies of
142 sequential or combination therapies of 5FUFA (bolus and infusion 5-FU with folinic acid)
143 with or without oxaliplatin or irinotecan as first- or second-line therapies in patients with
144 newly-diagnosed advanced CRC (16). A total of 2135 patients were recruited between 2000-
145 03 and randomised between three strategies of first- or second-line combination therapy.
146 Control strategy: First-line 5FUFA alone, followed by single-agent irinotecan; second
147 strategy: first-line 5FUFA alone, followed by second-line combination chemotherapy; third
148 strategy: combination chemotherapy in first line treatment. Within the two research
149 strategies, the combination regimen was an additional randomisation: either 5FUFA plus
150 oxaliplatin (FOLFOX), or 5FUFA plus irinotecan (FOLFIRI). For the DDIR analysis, samples
151 from patients with colonic primaries from a biobank of archival diagnostic tissue were
152 selected from consenting patients in the relevant arms where a randomised comparison
153 could be made between first-line 5FUFA alone or in combination with oxaliplatin (85mg/m²
154 two-weekly) (Supplementary Figure 1A). 385 samples were obtained from 371 primary
155 resections, 8 primary biopsies, 6 metastatic samples (3 liver, 2 nodal and 1 lung). The

156 primary outcome for FOCUS was overall survival (OS), but data were also available for
157 progression-free survival (PFS) and objective response rate (ORR).

158

159 ***FOxTROT Trial***

160 FOxTROT was an international randomised trial (1052 patients) which has reported its main
161 finding (7). Patients were eligible if they had been diagnosed with locally advanced colon
162 cancer (CC) without evidence of distance metastasis and with surgical resection of the
163 primary tumour planned. Patients were randomised into one of three chemotherapy
164 groups:

165 Group A: Patients had 6-weeks pre-surgery chemotherapy (oxaliplatin with either 5FUFA or
166 capecitabine) and 18-weeks chemotherapy that commenced 4-8 weeks after surgical
167 resection of the tumour.

168 Group B: Patients had no pre-surgery chemotherapy but had 24-weeks chemotherapy
169 (OxMdG or OxCap) after their surgical resection.

170 Group C: For patients who were RAS wild-type on baseline biopsy and randomised to neo-
171 adjuvant chemotherapy, the option of a secondary randomisation between panitumumab
172 or not, for the 6 weeks prior to surgery.

173 For patients randomised into Group A, FOxTROT provided an opportunity to measure DDIR
174 in the tissue biopsy in a subset at baseline and determine whether DDIR was predictive of
175 response to neo-adjuvant OxMdG therapy prior to resection surgery, excluding patients in
176 Group C and those with complete response (Supplementary Figure 1B).

177

178 **Gene Expression Profiling**

179 All the archival formalin-fixed paraffin-embedded (FFPE) tumour tissue samples were tested
180 at Almac's Diagnostic CLIA Laboratories. Samples were reviewed and tumour material
181 identified on an adjacent H&E stained slide for microdissection. Total RNA was extracted
182 from two sequential 5µm sections using the Roche High Pure FFPE Extraction Kit (Roche Life
183 Sciences, Penzberg, Germany) and amplified using the NuGen Ovation FFPE Amplification
184 System v3 (NuGen San Carlos, California, USA). The amplified product was hybridised to the
185 Almac Diagnostics XCEL array (Almac, Craigavon, UK), a cDNA microarray-based technology
186 optimised for archival FFPE tissue, and analysed using the Affymetrix Genechip 3000 7G
187 scanner (Affymetrix, Santa Clara, California, USA) as previously described (12). Microarray
188 data were quality checked (see Supplementary methods) then pre-processed where raw CEL
189 files underwent the Robust Multiarray Average (RMA) normalisation for the Almac
190 Diagnostic XCEL array with the affy package (v1.56.0) (17). Gene expression profiles from a
191 total of 391 samples from FOCUS and 97 samples from FOxTROT were made available.

192

193 For the biological analysis, a subset of gene expression profiles from n=361 primary tumour
194 resection samples from FOCUS were used (exclusions detailed in supplementary Figure 1A)
195 and n=97 pre-treatment biopsy samples from FOxTROT (exclusions detailed in
196 supplementary Figure 1B). Probes were annotated using annotation file "Xcel Annotations,
197 CSV format, Release 36" available for download from
198 (<http://www.affymetrix.com/support/technical/byproduct.affx?product=xcel>), and then
199 collapsed to their corresponding genes using WGCNA package (version 1.68), based on the
200 probe with highest average value for each gene (18). For comparative analysis between BC
201 and CRC, TRASNBIG BC cohort (19) containing gene expression profiles for 198 fresh frozen
202 samples from patients with node-negative T1-T2 (≤ 5 cm) breast performed on Affymetrix

203 Human Genome U133A array was downloaded from Gene Omnibus Expression (GEO;
204 www.ncbi.nlm.nih.gov/geo/) (accession number 'GSE7390').

205

206 **DDIR Signature**

207 A total of 484 clinical samples (391 from FOCUS and 97 from FOxTROT) had DDIR signature
208 scores calculated and predefined cut-points applied. The pre-defined threshold of 0.1094
209 was optimised in an independent technical study of 260 CRC samples whereby the optimal
210 threshold was detected at the score where the sensitivity and specificity meant a joint
211 maximum to accurately detect the DDIR-positive subgroup as defined in hierarchical
212 clustering (Personal communication Almac Diagnostics). The threshold was then applied
213 independently to the validation cohorts, dichotomising patients as DDIR-positive (>0.1094)
214 or DDIR-negative (≤ 0.1094).

215 TRANSBIG BC cohort (19) used in the original study had information available on
216 predetermined DDIR threshold of 0.37 along with DDIR continuous score (12), that was used
217 on our analysis.

218

219 **Consensus Molecular Subtyping and CRC Intrinsic Subtyping**

220 To obtain CMS calls, genes with multiple probesets were collapsed by mean and the
221 CMSclassifier package was used (20). Classification by random forest with the default
222 posterior probability of 0.5 showed a higher frequency of unclassified samples compared to
223 the original publication (20). To derive calls with comparable frequencies, single sample
224 predictor calls were computed after row-centring the expression data. Final CMS calls were
225 generated when there was a match between both methods without applying any cut-off. To
226 obtain CRIS calls, probesets with the highest average levels for each gene were selected and

227 the CRISclassifier package was used (21). Samples with a Benjamini-Hochberg-corrected
228 False Discovery Rate (BH.FDR) > 0.2 were left unclassified as originally reported (21).

229

230 **Mutational Analysis**

231 Mutation data was generated by DNA target capture (SureSelect, Agilent) spanning all
232 coding exons of 80 CRC driver genes (listed in Supplementary Methods) followed by next
233 generation sequencing (Illumina). Variant calling was performed with Caveman for point
234 mutations and Pindel for indel mutations. Driver mutations in *KRAS*, *NRAS*, *PIK3CA* and *TP53*
235 were considered for binary classification (e.g. depending on whether genes are
236 dominant/recessive, mutations reported as recurrent or an internal curated list) based on
237 frequency and relevance. *BRAF* was classified as mutated only with a V600E mutation.
238 Tumours showing more than two mutations in n=123 MSI markers within the panel were
239 classified as MSI, otherwise as MSS. The FOxTROT cohort showed a high failure rate (55/97
240 missing data, 57%) due to lack of enough tissue in small biopsies after RNA profiling.
241 Therefore, MSI classification from additional FOxTROT tumours were derived with a RNA
242 signature (22). Two borderline tumours were not classified.

243

244 **Gene Set Enrichment Analysis (GSEA)**

245 GSEA was performed in the three cohorts to investigate biological pathways associated with
246 DDIR (23,24), using Hallmarks gene set collection (h.all.v6.2.symbols.gmt [Hallmarks]) from
247 Molecular Signature Database (MSigDB) (25,26). GSEA version 19.0.26 was accessed from
248 the GenePattern cloud server web interface: <https://cloud.genepattern.org>. All default
249 parameters were utilised, with the exception of 'collapse dataset' which was set to 'FALSE',
250 as the probes were collapsed to their genes a priori, and the random seed was stated to be

251 '40218336'. Normal enrichment score (NES) and false discovery rate (FDR) values were
252 noted for each gene set within the two phenotypic (DDIR) groups, where FDR q-value below
253 25% was justified to be a significant gene set.

254

255 **Microenvironment Cell Population Analysis**

256 The MCPcounter (version MCPcounter_1.1.0) R package was downloaded from GitHub
257 (<https://github.com/ebecht/MCPcounter>), and was used to generate MCP estimation scores
258 for ten stromal and immune cell infiltrates from the transcriptomic data of the three cohorts
259 (27). Estimates were compared between DDIR-positive and DDIR-negative to determine
260 their stromal/immune content, and the differences in cellular composition between the
261 cancer types.

262

263 **Differential Gene Expression and Pathway Analysis**

264 Partek Genomics Suite (PGS) version 6.6 was utilised to perform ANOVA analysis to identify
265 differentially expressed genes with FDR of < 0.05, and fold change (FC) adjusted to 1.5 for
266 FOCUS and FOxTROT cohorts; for TRANSBIG due to the large number of differentially
267 expressed genes, FC value was increased to 2.5. Differentially expressed genes were
268 assessed using Ingenuity Pathway Analysis (IPA - 49932394) to examine any significant
269 biological pathways associated with DDIR subtypes. All parameters were set to default.

270

271 **Statistical Analysis**

272 Statistical analyses were conducted according to pre-specified statistical analysis plans that
273 were agreed prior to inspection of any DDIR-stratified outcome data. All clinical-related
274 analyses for Objective response rate, progression-free-survival and overall survival were

275 performed using Stat version 15.0 (Stata Corporation, Texas City, USA) or R (version 3.4.1).
276 Further detailed statistical analysis on FOCUS and FOxTROT cohort is available in
277 Supplementary Methods.

278

279 All statistical analyses undertaken for further biological exploration, including Pearson's
280 Correlation Coefficient, Fisher's exact test, Student's t-test, Wilcoxon rank sum test, Kruskal-
281 Wallis rank sum test, and one-way ANOVA followed by Tukey's Honest Significance
282 Difference test were performed to generate p-values for statistical significance using R stats
283 package in R (version 3.4.0) and RStudio (version 1.1383). In addition to base R packages,
284 *ggplot2* R package (version 3.2.1) with other supporting packages, including *cowplot*
285 (version 0.9.4), *ggpubr* (version 0.2.3) and *grid* (version 3.4.0) were used for graphical
286 visualisation.

287

288 **Data and Script Availability**

289 FOCUS and FOxTROT gene expression dataset and clinicopathological information are
290 provided from S:CORT, with transcriptional data available on GEO under reference
291 **GSExxxxxx (TBC)**. All scripts required to reproduce figures in this manuscript are available
292 from corresponding author on request or from www.dunne-lab.com.

293

294

295

296 **Results**

297

298 ***Case selection from FOCUS metastatic CRC clinical trial***

299 A total of n=391 patients were available for DDIR analysis from the FOCUS trial. Following
300 exclusion of rectal cancer cases and prioritisation of resected tissue to ensure there was
301 sufficient tumour tissue for molecular analyses, n=310 from the 5FU alone group and n=81
302 in the 5FU+oxaliplatin group were used for outcome analyses (Supplementary Table S1).

303 Assessment of baseline characteristics of patients excluded from the DDIR analysis
304 compared to those included in the DDIR analysis revealed that there were no other obvious
305 selection biases between the groups (Supplementary Table S1, Supplementary figure S1). A
306 total of 76/391 patients were classified as DDIR positive (Supplementary Figure S2),
307 generating a prevalence of 19% [95% CI 16-24] overall, with a reasonable balance between
308 the randomised groups of 63 (20%) versus 13 (16%) in the 5FU and 5FU+oxaliplatin groups
309 respectively, (Chi-squared p-value for difference=0.39; Supplementary Table S1).

310 The overall prevalence of DDIR was lower than anticipated when compared with data from
311 other cohorts of patients with CRC (28) and other disease indications (12,13,29) but was
312 similar to the technical study of 260 metastatic CRC used to set the threshold for DDIR
313 positivity (Personal communication Almacgroup).

314

315 ***Survival analyses according to DDIR status in the FOCUS trial***

316 During the course of follow-up between 16th May 2000 and 18th October 2006, there were a
317 total of 383 PFS events (357 during the first 15 months) and 342 OS events. During the first
318 12-weeks of first-line chemotherapy, there were 157 (40%) complete or partial responders
319 and 234 (60%) stable or progressive disease non-responders. A comparison between
320 randomised groups, without stratification for DDIR, confirmed the anticipated treatment
321 effect of oxaliplatin; PFS adjusted HR (95% CI) = 0.63 (0.48, 0.82), p=0.001 and ORR adjusted
322 OR (95% CI) = 4.11 (2.37, 7.14), p<0.001 (data not shown).

323

324 In the FOCUS control arm, we identified no prognostic effect of DDIR status for patients with
325 metastatic colon cancer treated with first line 5FU alone, either on OS (Unadjusted HR (95%
326 CI) = 0.95 (0.71, 1.28), $p = 0.73$, Test of proportional hazards: $\chi^2 = 1.42$ on 1 d.f., $p=0.20$,
327 Supplementary Figure S2b), or on PFS (Adjusted HR = 1.11 (95% CI 0.79 – 1.54), $p = 0.55$).
328 This result remained non-significant when adjusted for clinical variables, CMS status and
329 other molecular variables.

330

331 Using fully adjusted models, we next explored the predictive effects of DDIR for all
332 outcomes, with PFS at 15 months as the primary outcome (Figure 1A). Contrary to the
333 expectation that DDIR-positive patients would derive the most benefit from oxaliplatin,
334 DDIR-negative patients appeared to respond more frequently to FOLFOX (ratio of odds
335 ratios for ORR = 0.15 (95% CI 0.04 – 0.65), test for interaction $p = 0.011$; Table 1, Figure 1B).
336 Although this inverted direction of effect was the same for the survival outcomes, the tests
337 for interaction were non-significant (Table 1).

338

339 ***Case selection and survival analyses according to DDIR in the FOxTROT neoadjuvant CRC*** 340 ***clinical trial***

341 Following these analyses in the metastatic setting, we next assessed the clinical utility of the
342 DDIR in the CRC neoadjuvant setting. A total of 97 patients who received neoadjuvant
343 FOLFOX were selected from Group A of the FOxTROT dataset. Patients were excluded if they
344 withdrew from the trial, if they did not receive neo-adjuvant chemotherapy or if they
345 received OxCap prior to surgery. Additionally, no patients with complete pathological
346 response were forwarded to S:CORT for analysis. These selections led to a somewhat biased
347 subset compared to the main study with less responders, less MSI and more KRAS wildtype

348 tumours (Supplementary Table 2). Of these 97 patients, 4 had no associated response data,
349 leaving a total of 93 patients who were included in the final analysis. There were a total of
350 40 non-responders, 29 mild-responders, 17 moderate responders and 7 marked responders.
351 The DDIR threshold was set at the same value defined in the FOCUS cohort, resulting in 57%
352 DDIR positive patients, which was considerably higher than the 19% seen in the metastatic
353 FOCUS dataset (Supplementary Figure S2c). Using ordinal regression across the 4 response
354 groups, there were marginally better responses in the DDIR-negative group (Figure 1C), but
355 this was not statistically significant using unadjusted ordinal regression OR = 0.62 [95% CI
356 0.29 – 1.33], p=0.218 (Table 1). After adjustment for age, sex, pT-stage, pN-stage, primary
357 tumour location, MSI and RAS status, the coefficient reduced slightly to 0.55 [95% CI 0.21-
358 1.39], p=0.205. Employing DDIR as a continuous variable, the unadjusted OR for response
359 was 0.19 [95% CI 0.02-1.79], p=0.148. When adjusted for age, sex, T-stage, N-stage,
360 left/right, MSI and RAS status the OR reduced to 0.11 [95% CI 0.01-1.66], p=0.110
361 (Supplementary Table S2).

362

363 Given these counter-intuitive findings, we next set out to investigate if there was a
364 biological explanation for this potentially inverted and inconsistent effect between previous
365 breast cohorts and our CRC trial cohorts.

366

367 ***Association between DDIR and colorectal cancer subtypes***

368 Investigation into the biological relevance of DDIR signature led to the comparison against
369 CRC Consensus Molecular Subtypes (CMS) which is largely based on histological (stroma and
370 immune) features (20). In the FOCUS cohort, immune-rich CMS1 tumours are significantly
371 associated with increased DDIR scores when compared to all other CMS subtypes (Figure

372 2A; Kruskal-Wallis, $p < 0.0001$). Despite CMS1 tumours having a significantly higher
373 proportion of DDIR-positive tumours compared to the other subtypes (Supplementary
374 Figure 5A; Fisher's exact test, $p = 0.0002$), given the low prevalence of DDIR-positivity across
375 the whole cohort, 68% of CMS1 subtypes are below the DDIR threshold (Figure 2A). Of note,
376 there are proportionally more CMS4 tumours within DDIR-negative classification in the
377 FOCUS cohort (Supplementary Figure 5A). In pre-treatment biopsies from the smaller
378 FOxTROT cohort, CMS1 tumours show a non-significant trend towards DDIR positivity
379 (Figure 2B; Kruskal-Wallis, $p = 0.4695$, and Supplementary Figure 5B; Fisher's exact test, $p =$
380 0.4879). Additionally, we also examined DDIR on Colorectal Cancer Intrinsic Subtypes (CRIS)
381 that represents CRC tumour-intrinsic (epithelial) biology (21). Contrary to CMS, no
382 significant association between the CRIS subtypes and DDIR-positive or DDIR-negative
383 tumours in both the FOCUS and FOxTROT cohort was found (Supplementary Figures 5C-F).
384 These findings suggest that, in CRC, DDIR-positivity is primarily associated with (and
385 potentially influenced by) CMS-related tumour microenvironment (TME) factors, such as
386 differences in stromal/immune infiltrates, rather than epithelial-derived intrinsic factors.

387

388 Originally, DDIR signature was developed based on defective DNA damage response and
389 repair machinery of Homologous Recombination (HR) and Fanconi Anaemia (FA) in breast
390 cancer (12). However, there is limited evidence on their role in CRC tumorigenesis (30).
391 Thus, we explored the relationship between HR/FA and DDIR in CRC cohorts and made
392 comparison against TRANSBIG BC cohort which was used in the development of the DDIR
393 signature. Our investigation suggested that within CRC, these pathways do not show any
394 association with DDIR, contrary to that in BC (see Supplementary Results; Supplementary
395 Figure 3). Microsatellite instability (MSI), a result of defective DNA mismatch repair

396 mechanisms, defines a proportion of CRC patients associated with high tumour mutational
397 burden, leading to development of immune-responsive TME. Despite the limited number of
398 MSI tumours in the metastatic FOCUS CRC cohort (n=13), we observe that MSI tumours
399 contain a significantly higher proportion of DDIR-positives (Figure 2C; Fisher's exact test, p =
400 0.0211). However, DDIR-positivity is not a biomarker of MSI status, as only 46% of MSI
401 tumours are DDIR-positive (6 out of 13) while the majority of DDIR-positive tumours overall
402 are MSS (Figure 2D; MSI/DDIR+ n=6, MSS/DDIR+ n=59). In the FOxTROT cohort, MSI trends
403 observed are in line with the larger FOCUS cohort (Figure 2E; Fisher's exact test, p = 0.2522,
404 and Figure 2F; Student's t-test, p = 0.0737), but this result cannot be used to confirm the
405 FOCUS findings due to small (n=3) MSI sample size (Figure 2F). Furthermore, while MSI
406 tumours collectively contain higher mutational burden than MSS as expected, mutational
407 burden is not associated with DDIR-positivity in either of the CRC cohorts (Supplementary
408 Figure 5G; Student's t-test, p = 0.1279 and Supplementary Figure 5H; Student's t-test, p =
409 0.4534).

410

411 ***Enhanced immune-related signalling pathways define DDIR-positive tumours***

412 To further characterise the biological functions and pathways associated with DDIR, we
413 performed GSEA, using the "Hallmark" collection, to compare DDIR-positive and DDIR-
414 negative tumours in FOCUS and FOxTROT CRC cohorts, compared to the same analyses in
415 the TRANSBIG BC cohort. GSEA between DDIR-positive and DDIR-negative tumours
416 generated different numbers of significant Hallmarks genesets in each cohorts
417 (Supplementary Figure 6). However, in general, between the three cohorts five common
418 significantly-enriched genesets in DDIR-positive CRC and BC tumours were identified,
419 namely allograft rejection, IL6/JAK/STAT3 signalling, inflammatory response, interferon- α

420 response and interferon- γ response (Figure 3A; FDR q-value < 0.25), suggesting that a
421 common immune and/or inflammatory-like signalling defines DDIR-positivity, regardless of
422 the cancer type. Interestingly, we also observe eight unique gene sets that are only
423 associated with DDIR in BC and not in CRC (Figure 3A).

424

425 Previous studies of DDIR signalling in BC have highlighted increased levels of the interferon
426 gamma-induced chemokine CXCL10 gene/protein expression in DDIR-positive tumour cells,
427 leading to lymphocytic trafficking into the tumour (14). Here, we showed that CXCL10
428 expression has a strong positive (>6) correlation with DDIR scores in both BC and CRC
429 cohorts (Figure 3B, 3C and 3D). Additionally, it was previously demonstrated that DDIR-
430 positivity in BC was specifically associated with activation of cGAS/STING/TBK1 innate
431 immune response axis (14). This, however, was not found to be the case in CRC (see
432 Supplementary Results).

433

434 ***DDIR-defined tumour microenvironment reflects immune-rich colorectal subtype***

435 We tested the association between immune/stromal composition, based on gene
436 expression profiles using microenvironment cell population (MCP) analysis, where we
437 identified consistent correlations between DDIR scores and T cell, B cell and monocytic
438 immune lineages, confirming an increase in lymphocytic infiltration in DDIR-positive BC
439 (Figure 4A; Pearson r; T cells = 0.7167, B Lineage = 0.5075, Monocytic Lineage = 0.7042).

440 While we also observe correlative trends in both CRC cohorts (Figure 4B; Pearson r; T cells =
441 0.3509, B Lineage = 0.2774, Monocytic Lineage = 0.2358 and Figure 4C; Pearson r; T cells =
442 0.4038 and Monocytic Lineage = 0.5152 and B Lineage, r = 0.3666), these correlations were
443 not as strong as those observed in BC. Moreover, cytotoxic lymphocytes scores also

444 demonstrate a positive correlation with DDIR using both a positive versus negative
445 categorical (Figure 4D; Student's t-test, $p < 0.0001$) or DDIR continuous score (Figure 4D;
446 Pearson $r = 0.6106$) in the TRANSBIG BC cohort. Similar, albeit weaker, correlations were
447 observed in both FOCUS (Figure 4E: Student's t-test, $p < 0.0001$; Pearson $r = 0.436$) and
448 FOxTROT (Figure 4F: Student's t-test, $p = 0.0004$; Pearson $r = 0.5251$) CRC cohorts using the
449 MCP-derived cytotoxic lymphocyte scores. Incorporation of CMS in the CRC analyses
450 demonstrated the association between CMS1, lymphocytic infiltration and increased DDIR
451 score. Levels of cytotoxic CD8⁺ T-lymphocytic infiltration were further assessed in situ in the
452 FOCUS cohort by IHC (Figure 4G), where a significant association between CD8 IHC scores
453 and DDIR score was observed, in line with MCP assessments in these tumours (Figure 4H:
454 Student's t-test, $p < 0.0001$; Pearson $r = 0.4388$). Conversely, fibroblast levels and CMS4
455 subtypes were negatively correlated with DDIR score in the FOCUS cohort (Supplementary
456 Figure 7A and 7B; t-test, $p = 0.0109$; Pearson $r = -0.1597$), while no association was noted in
457 FOxTROT cohort (Supplementary Figure 7C and 3D: t-test, $p = 0.9984$; Pearson $r = 0.0291$).

458

459 ***Overlapping interferon-responsive biology in DDIR-positive CRC and BC***

460 Next, we set out to identify overlapping individual differentially expressed genes between
461 DDIR subtypes in both BC and CRC. Differential gene expression analysis comparing DDIR-
462 positive and DDIR-negative tumours identified 66 and 60 differentially expressed genes in
463 FOCUS and FOxTROT cohorts respectively (FDR < 0.05 , FC = 1.5; Figure 5A). We observed
464 975 differential genes between DDIR-positive and negative tumours in the BC cohort
465 compared to CRC; thus, in order to limit these analyses to a similar sized gene list for the
466 TRANSBIG cohort, we increased the FC for analysis, identifying 110 differentially expressed
467 genes (FDR < 0.05 , FC = 2.5; Figure 5A). Comparison of gene lists from the three cohorts

468 identified nine genes that are consistently upregulated in DDIR-positive tumours in both
469 cancer types (Figure 5A). This list contained members of chemokines family, including two
470 genes (CXCL10 and IDO1) that are part of the 44-gene DDIR signature. Using these nine
471 differentially expressed genes common in all three cohorts, pathway analysis was
472 performed, which revealed 18 potential upstream regulators of conserved biology
473 contributing to DDIR-positivity across CRC and BC, including key regulators of inflammatory
474 and interferon-related signalling; such as IFN-alpha, IFN-gamma, STAT1 and the NFkB
475 complex (Figure 5B and Supplementary Figure 8A).

476

477 Using these nine consensus DDIR-related genes to generate an unweighted cumulative
478 score, we observed a strong positive correlation between this new overlapping ranked sum
479 score and the original DDIR score (Figure 5C; Pearson $r = 0.6291$, $p < 0.0001$). In line with
480 this overlap, we also observed similar correlative trends for both CMS and MSI
481 (Supplementary Figure 8B and 8C), with the nine gene score as observed with the original
482 DDIR score (Figure 2). Finally, a Cox regression model (for PFS) and a logistic regression
483 model (for response) were fitted with main effects for oxaliplatin and for each of three
484 quartiles of Almac DDIR or 9-gene score relative to Q1 (reference), and interactions
485 between oxaliplatin and the three quartiles (Figure 5D). As with the response and outcomes
486 analyses using the original DDIR score, this overlapping nine gene score fails to predict a
487 benefit for the addition of oxaliplatin to 5FU in the FOCUS trial. Importantly, however, this
488 new refined CRC DDIR signature removes the trend for increased response to oxaliplatin
489 observed in the DDIR-negative group in the original DDIR.

490

491 **Word Count: 2255**

492 **Discussion**

493

494 The original characterisation of the DDIR signature demonstrated its predictive value as a
495 biomarker for platinum-based chemotherapy treatment in BC, and subsequently
496 oesophageal adenocarcinoma (OAC) (12,29). In the initial BC study, the biology
497 underpinning DDIR was based on dysfunctional DNA damage response and repair machinery
498 regulated via the HR and FA/BRCA pathways, which is targeted by some chemotherapies as
499 a mode of action (31). The multi-disciplinary S:CORT consortium (15) was established to
500 identify and test new molecular stratification methods to predict CRC response to
501 treatments, through the discovery of new and/or validation of existing molecular
502 biomarker-based assays. In this study, we tested the clinical utility of the 44-gene DDIR
503 signature from archival FFPE tumour tissue profiled at Almac's Diagnostic CLIA Laboratories
504 as previously described, to predict response to the addition of oxaliplatin to 5-FU-based
505 chemotherapy in both metastatic CRC (FOCUS cohort) and neoadjuvant CRC (FOxTROT)
506 clinical trial settings. Accompanying this clinical assessment, we utilised the molecular and
507 histological data generated to further interrogate the biological signalling associated with
508 CRC-specific DDIR positivity in contrast to BC.

509

510 DDIR-positivity was observed in 19% of primary tumours from stage IV FOCUS cohort and
511 57% of primary tumour biopsy material from stage II/III FOxTROT cohort. A previous study
512 of DDIR-positivity in CRC reported a 35% incidence in a predominantly (94%) non-metastatic
513 population (28). This was comparable to findings in BC (34%) (12) and OAC (24%) (29).
514 Differing DDIR rates in our study could be credited to the cancer stage or other (molecular)
515 criteria used for patient selection in the original trials. Patients with localised disease, as in

516 the neo-adjuvant FOxTROT study, have a higher proportion of tumours with immune
517 infiltration (32), a factor associated with DDIR-positivity in BC and OAC, and also with MSI
518 and CMS1 tumours in CRC. Similarly, the reduction in DDIR-positivity to ~20% in metastatic
519 disease is consistent with a lower relative proportion of patients with MSI in metastatic
520 disease, which falls from ~20% in localised CC in ~4% in mCRC, as in the FOCUS cohort.

521

522 MSI is the most notable feature in CRC displaying defective DNA damage response and
523 repair via mismatch repair (MMR) system (30). MSI and CMS1 are closely linked together
524 with high tumour mutation burden, overproduction of tumour-specific neoantigens,
525 increased immune infiltration and show favourable clinical outcome in early stage disease
526 (20). Given their high levels of immune infiltration and mutation burden, these tumours
527 have responded well to checkpoint blockade immune-oncology (IO) treatments (33). There
528 is a strong association of DDIR status with CMS1, MSI status (28) (Figure 2) in FOCUS cohort,
529 and a similar trend is observed in FOxTROT cohort, given its small sample size (Figure 2),
530 reflecting the observed clinical utility of immunotherapeutic interventions in this molecular
531 subtype (34,35). However, our findings do not validate the correlation between DDIR and
532 mutational burden in the FOCUS cohort observed in the CRC threshold development
533 abstract (28), likely due to the difference in disease stage (FOCUS as mCRC) and mutational
534 panel sequencing methods used with S:CORT.

535

536 Contrary to our primary hypothesis, it was noted that response to the addition of oxaliplatin
537 to 5FUFA was more likely to benefit DDIR-negative patients in both FOCUS and FOxTROT
538 cohorts rather than DDIR-positive patients. While this was only statistically significant in
539 terms of response in the metastatic FOCUS trial setting (ratio of odds ratios for ORR = 0.15,

540 test for interaction $p = 0.011$), the trend was consistent across all endpoints in both cohorts
541 examined. However, the refinement of DDIR gene signature to only 9-genes signature
542 through our analysis showed no additional benefit from oxaliplatin for either DDIR-positive
543 or DDIR-negative patients (Figure 5). The original and subsequent DDIR study in BC with the
544 South Western Oncology Group (13) demonstrated improved response to anthracycline
545 and/or cyclophosphamide-based neoadjuvant and adjuvant chemotherapy in DDIR-
546 positive patients. Similarly, in OAC, DDIR-positivity was predictive of improved response to
547 cisplatin-containing chemotherapy (29). Oxaliplatin is known to differ in its mechanism of
548 cytotoxicity compared to cisplatin and may have more complex mode of action in CRC (36).

549

550 Although we show no additional interaction between DDIR-positivity and oxaliplatin
551 treatment, biologically, our study highlights promising immunotherapeutic opportunities
552 among DDIR-positive CRC patients, beyond the use of general immune infiltration or MSI
553 status. DDIR-positivity may have value in identifying additional subsets of MSS CRC patients
554 who exhibit high tumour mutational burden and/or high TME activity, who have the
555 potential to respond to immune checkpoint blockade such as PD-L1 inhibition (35,42,43).
556 The search for biomarkers to distinguish immune “cold” tumours (that display limited
557 response to IO) from immune “hot” tumours (that respond to IO) has gained traction in
558 recent years. Our findings indicate that in CRC, although DDIR-positivity is associated with
559 increased levels of both innate and cytotoxic infiltration, likely to be driven by interferon-
560 related signalling, the immune system is in an “exhausted” state and unable to efficiently
561 clear these tumours, due to the concurrent expression of checkpoints such as IDO1 and PD-
562 L1 (CD274) (Figure 6E). These findings may also provide an explanation for the non-
563 correlation of DDIR with oxaliplatin-based chemotherapy response, as induction of immune

564 tolerance is a common response pattern to inflammation in the gut and tumour-associated
565 inflammation (as seen in DDIR positive tumours) that leads to a predominantly immune
566 suppressive milieu, which is further reinforced by additional chemotherapy-related
567 inflammatory signalling. Indeed, MSI tumours are largely non-responsive to chemotherapy,
568 as has been demonstrated recently in the neoadjuvant FOxTROT trial (7), as are immune-
569 rich/MSI tumours when assessed in other non-trial adjuvant cohorts (44). Very recent trial
570 data reported 100% response rate in early-stage MSI CC, including 60% pathological
571 complete response, to neoadjuvant IO treatment (combined CTLA-4 and PD1 blockade) (45).
572 Results from that study also indicate that only 27% of MSS tumours displayed any response.
573 Importantly, however, these data confirmed the predictive nature of CD8⁺ T cell infiltration
574 for IO response in MSS tumours; a phenotype associated with the biology underpinning
575 DDIR-positivity in MSS CRC presented in this study, supporting clinical testing of DDIR as a
576 predictive assay to select MSS patients in this setting.

577

578 The approach adopted in our study highlights the clinical utility and high success rates
579 associated with molecular profiling of FFPE material (Supplementary Table 1), even in tissue-
580 limited pre-treatment diagnostic biopsy material used to guide treatment decisions in the
581 neoadjuvant setting, as in FOxTROT. The TRANSBIG data used in the original DDIR study
582 poses a potential limitation on our BC analysis due to the platform employed in the original
583 analysis (Affymetrix Human Genome U133A Array) not being identical to the one used for
584 the transcriptional profiling in the CRC cohorts, which was the Almac XCEL array. To ensure
585 cross-platform comparison for DDIR was not confounding our study, Almac have classified
586 DDIR according to their diagnostic assay on all cohorts tested.

587

588 In summary, our study shows that, in contrast to BC and OAC, DDIR does not predict
589 improved response or survival to oxaliplatin treatment. We have identified the underlying
590 biology of the signalling associated with DDIR in CRC that could effect the outcome. While
591 we identify significant overlap in DDIR signalling across BC and CRC, particularly immune-
592 related TME signalling, we also highlight that signalling associated with both HR/BRCA and
593 STING pathways is not significantly associated with DDIR in CRC. Overall, our data supports
594 further testing of the utility of the DDIR signature in selecting patients who may respond to
595 IO-based therapy.

596

597 **Word Count: 1226**

598

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605

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611 Paddy Johnston from Queen's University Belfast. Sadly, soon after the project commenced

612 Paddy passed away and we would like to dedicate this work to him.

613

614

615

616

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Figure 1

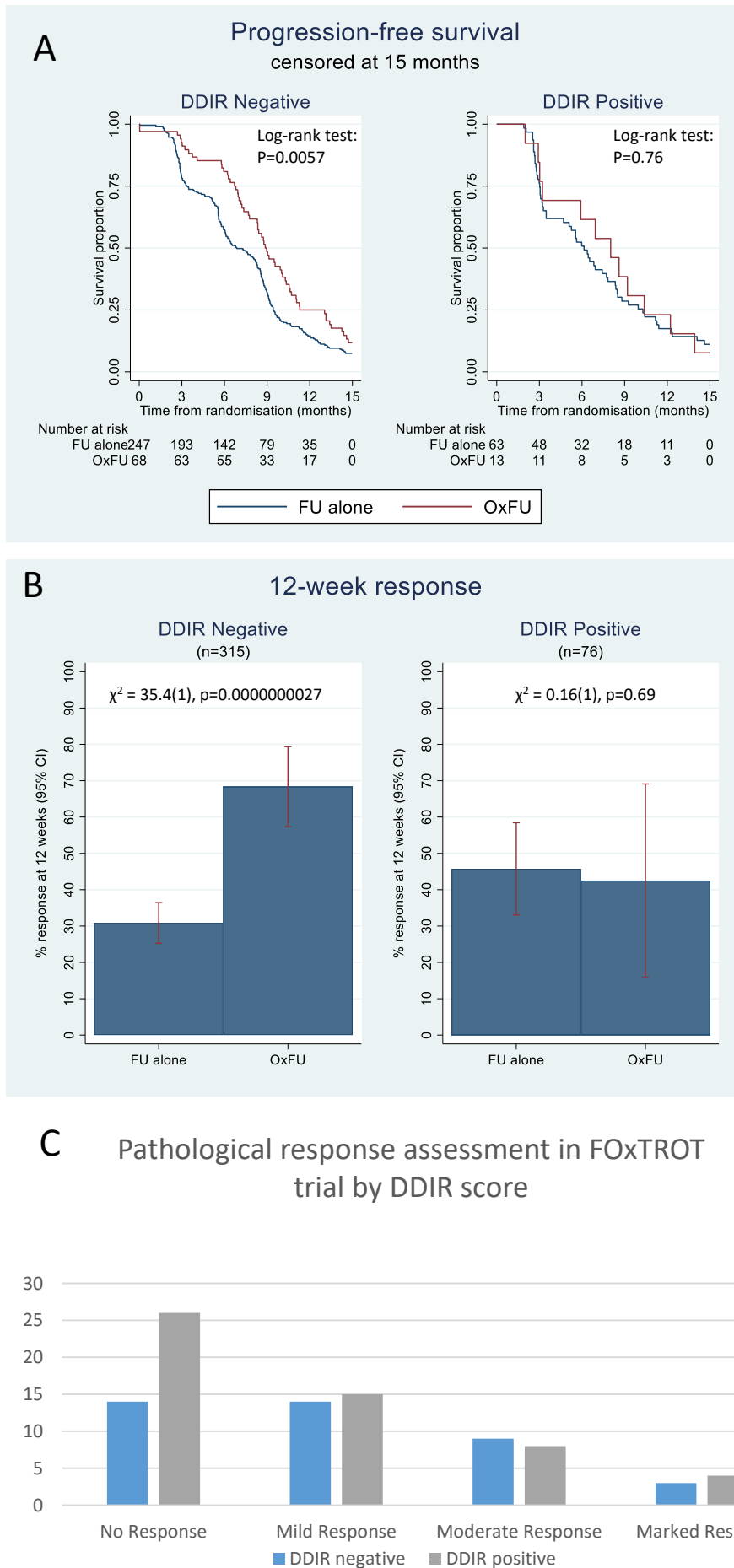


Figure 1. Clinical outcomes in patients randomised to FUFA or to OxFU in FOCUS trial by DDIR score. A. Progression free survival (to 15 months) B. Overall response rate (ORR) C. Pathological response assessment in resected primary following 6 weeks oxaliplatin based chemotherapy in FOxTROT trial by DDIR score.

Table 1

	DDIR negative (81%)		DDIR positive (19%)			
Outcome (FOCUS)	HR or OR for OxFU vs 5FU alone	(95% CI) p-value	HR or OR for OxFU vs 5FU alone	(95% CI) p-value	Interaction HR or OR	(95% CI) p-value
PFS (15 months)	0.59	(0.44, 0.80) P=0.001	0.85	(0.45, 1.62) P=0.63	1.43	(0.70, 2.92) P=0.32
PFS (Full)	0.58	(0.43, 0.76) P<0.001	1.00	(0.54, 1.87) P=0.99	1.73	(0.87, 3.43) P=0.12
OS (Full)	0.88	(0.65, 1.18) P=0.38	1.26	(0.65, 2.46) P=0.50	1.44	(0.69, 3.01) P=0.34
ORR	5.64	(3.01, 10.56) P<0.001	0.86	(0.23, 3.16) P=0.82	0.15	(0.04, 0.65) P=0.011

	DDIR negative (41%)		DDIR positive (59%)			
Outcome (FoxTrot) ORR	N	%	N	%	Unadjusted ordinal regression	(95% CI) p-value
excel	14	35%	26	49%	0.62	(0.29, 1.33) P=0.128
Mild Response	14	35%	15	28%		
Moderate Response	9	23%	8	15%		
Marked Response	3	7%	4	8%		

Statistical outcomes to oxaliplatin based therapy by DDIR status in 1. FOCUS trial and 2. FoxTROT trial sample sets

Figure 2

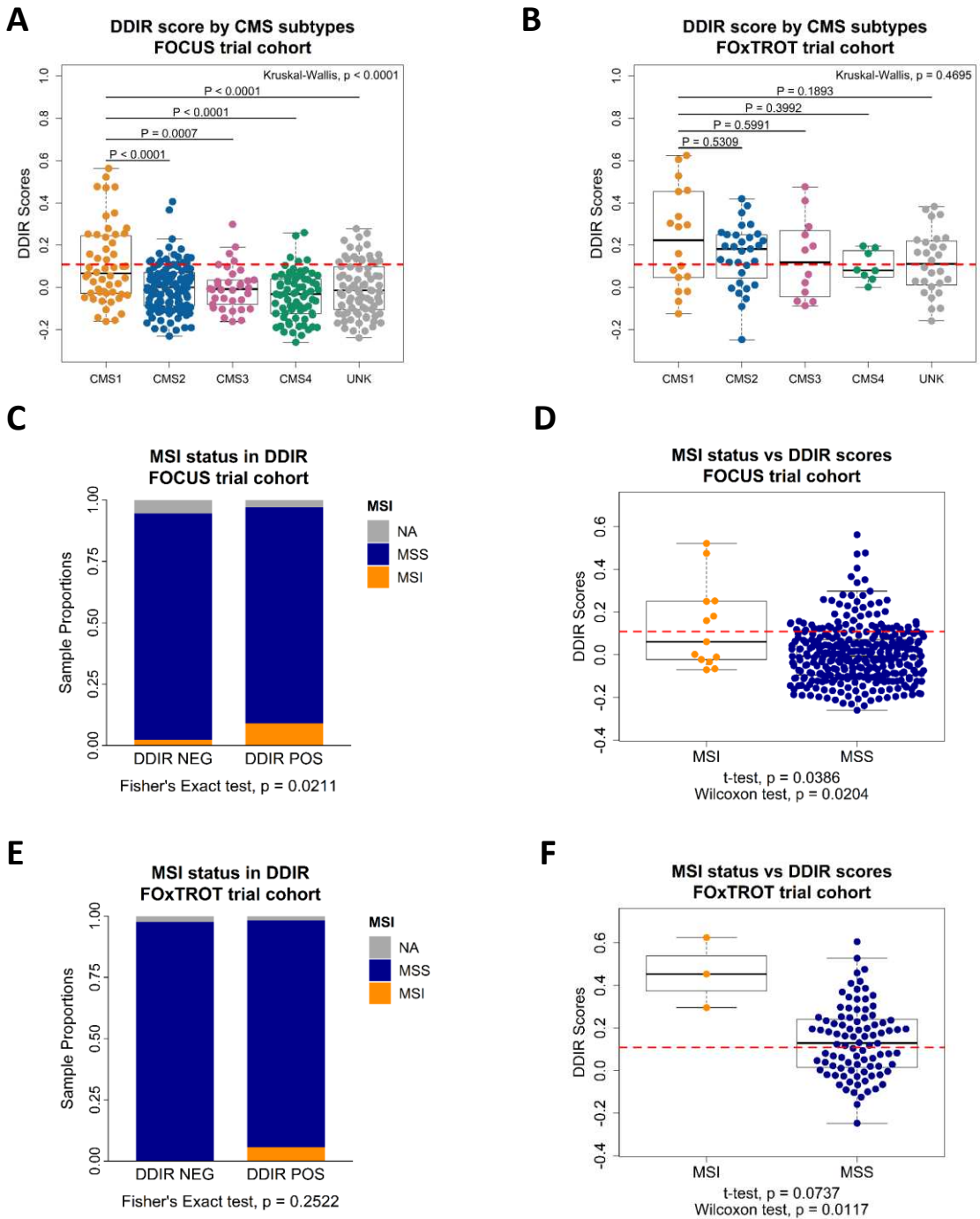
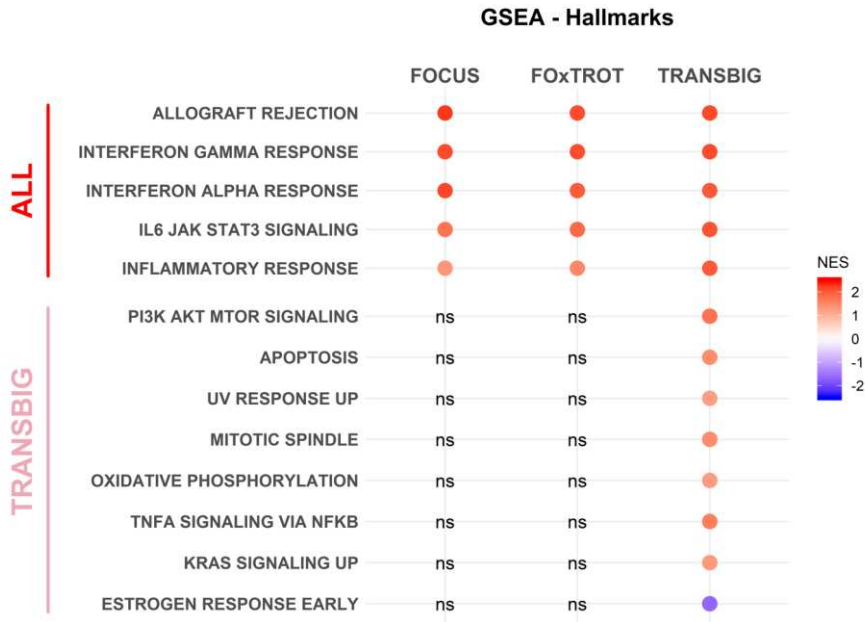


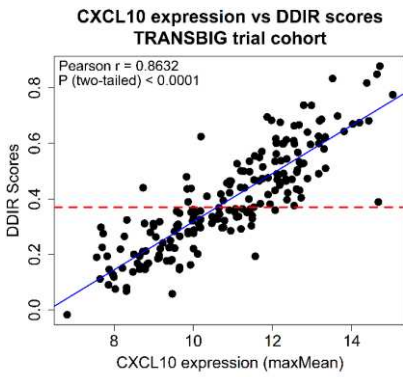
Figure 2. Consensus molecular subtypes (CMS) and CRC intrinsic subtypes (CRIS) in association with DDIR in adjuvant FOCUS and neoadjuvant FOxTROT clinical trial cohorts. **A)** Distribution of CMS samples against DDIR score in FOCUS and **B)** FOxTROT cohort, shown with DDIR threshold value at 0.1094 (red dash line). Statistics: Kruskal-Wallis rank sum test for global p -value, and Tukey's HSD test following one-way ANOVA for comparison between two groups. **C)** Proportion of MSI/MSS CRCs in the FOCUS cohort comparing DDIR positive and DDIR negative, and **D)** number of MSI/MSS CRCs in the FOCUS cohort samples against DDIR continuous score. **E)** Proportion of MSI/MSS CRCs in the FOxTROT cohort comparing DDIR-positive and DDIR-negative, and **F)** number of MSI/MSS CRCs in the FOxTROT cohort samples against DDIR continuous score. Statistics: Pearson's Coefficient Correlation, Fisher's exact test, Student's t -test and Wilcoxon rank sum test.

Figure 3

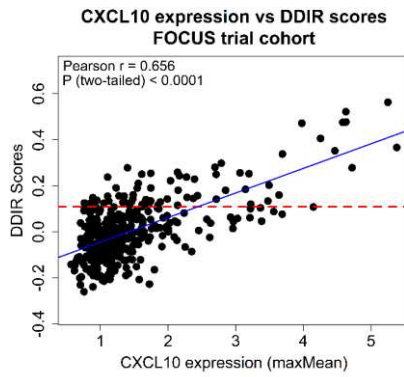
A



B



C



D

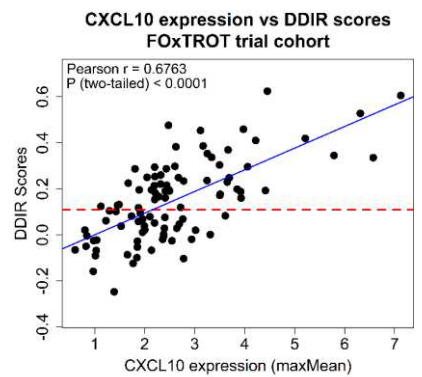


Figure 3. Inflammatory and immune response-related pathways are elevated in DDIR positive tumours. **A)** Gene set enrichment analysis on the two CRC cohorts (FOCUS and FOxTROT) and a BC cohort (TRANSBIG) identifies five common pathways associated with DDIR positive tumours in both cancer types; Benjamini-Hochberg False Discovery Rate (FDR) < 0.25 considered significant, Normalised Enrichment Score (NES) bar (DDIR POS > 0 , DDIR NEG < 0). **B)** Expression of CXCL10 correlated with DDIR scores in TRANSBIG, **C)** FOCUS, and **D)** FOxTROT cohort, displayed with line of best fit (blue).

Figure 4

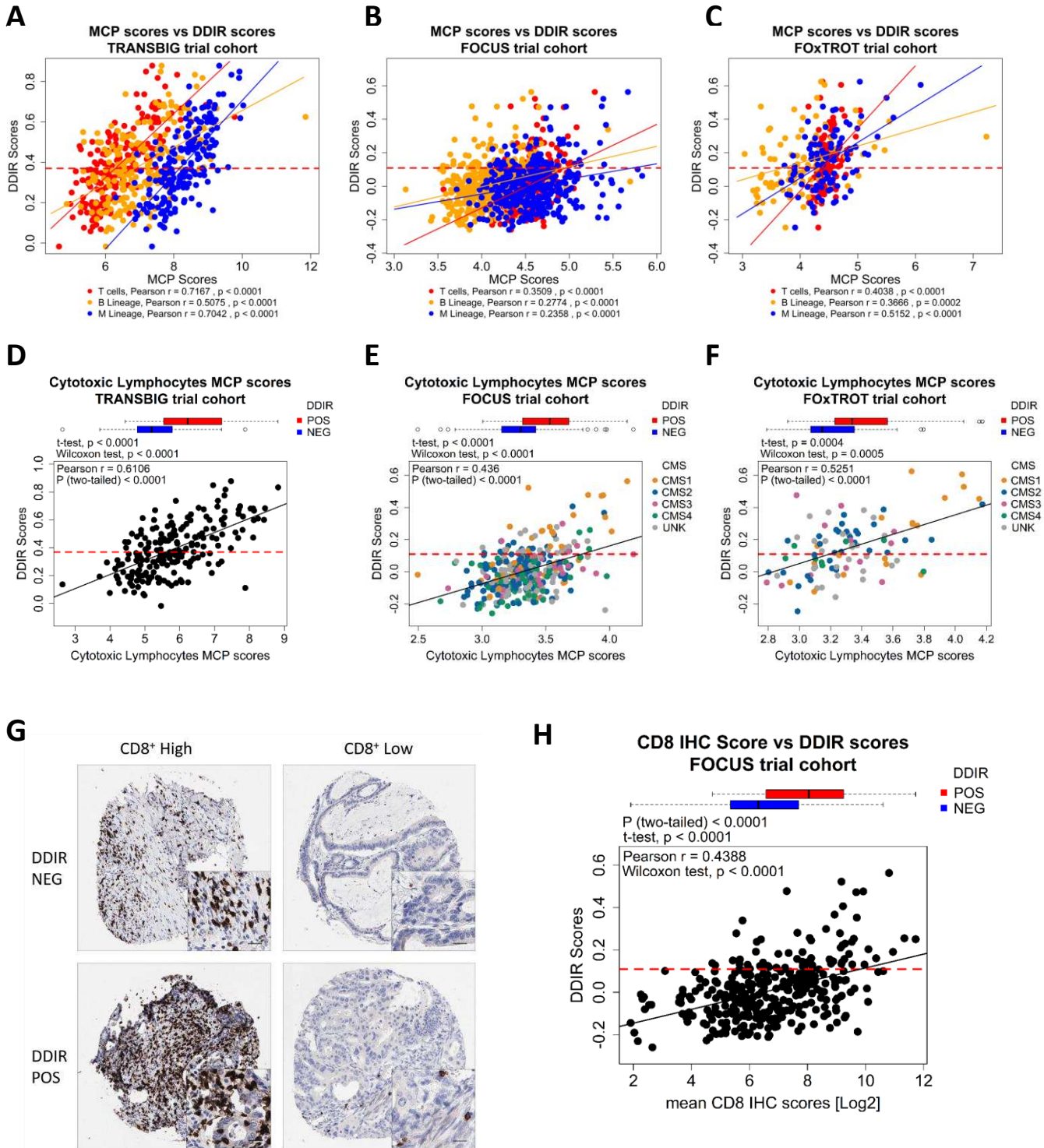


Figure 4. Increased immune infiltrates highly correlates with DDIR positivity. **A)** MCP scores of three immune infiltrates – T cells (red), B lineage (yellow) and monocytic lineage (blue) – correlated against DDIR scores with line of best fit for each immune infiltrates for TRANSBIG, **B)** FOCUS, and **C)** FOxTROT cohort.; shown DDIR threshold value at 0.37 for BC and 0.1094 for two CRC cohorts (red dash line). **D)** Cytotoxic lymphocytes MCP scores correlated with DDIR score in TRANSBIG, **E)** with overlay of CMS in FOCUS, and **F)** FOxTROT cohort; shown DDIR threshold value at 0.37 for BC and 0.1094 for two CRC cohorts (red dash line). **G)** Immunohistochemistry (IHC) images of DDIR negative and DDIR positive tumours stained with CD8⁺ marker in FOCUS cohort (x10; inset x40, 20 μ m bar). **H)** Comparison of average CD8⁺ log-transformed scores from IHC analysis between DDIR positive (red) and DDIR negative (blue) shown in boxplot above scatterplot examining correlation with DDIR continuous score; line of best fit (black) and DDIR threshold value at 0.1094 (red dash line). Statistics: Student's *t*-test, Wilcoxon rank sum test and Pearson's Coefficient Correlation.

Figure 5

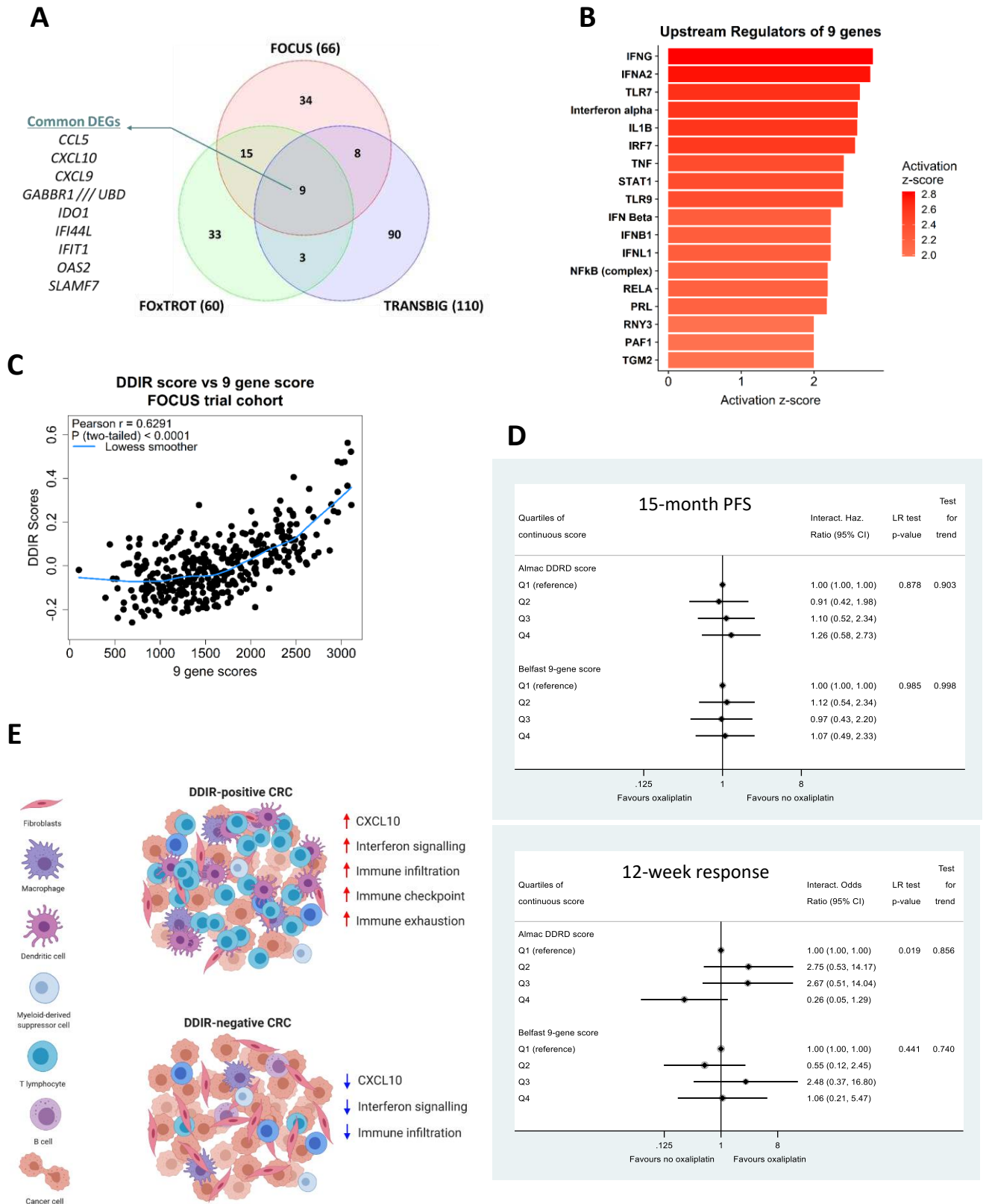
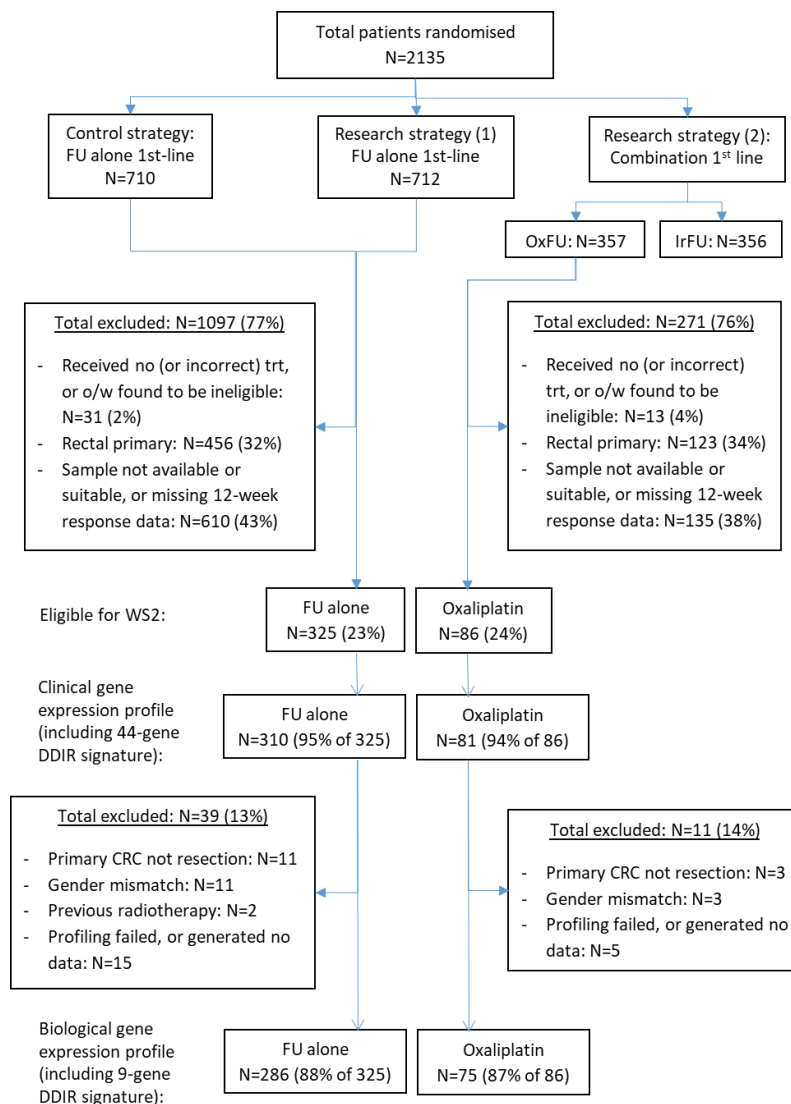


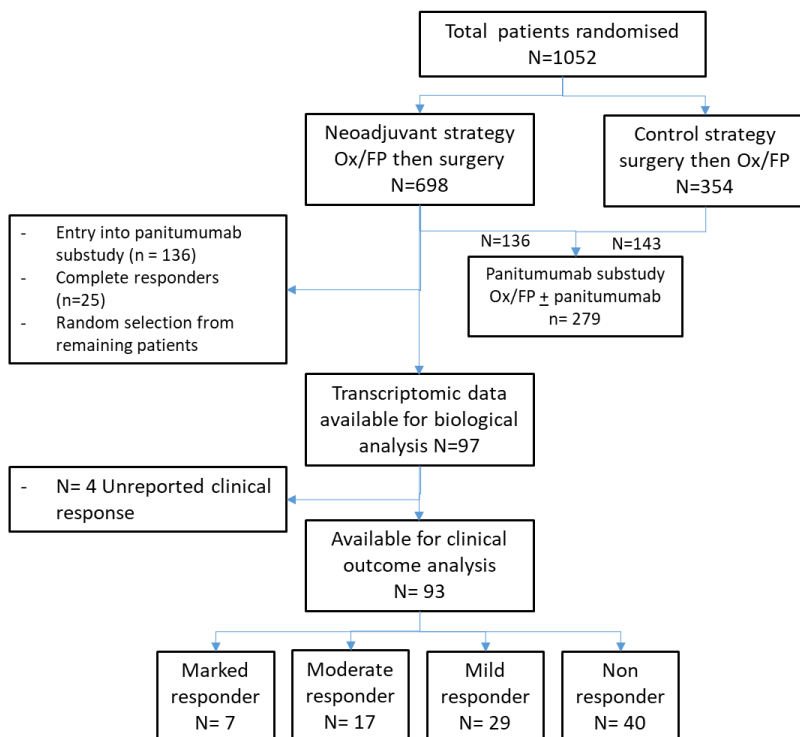
Figure 5. Differential gene expression analysis identifies distinct and conserved DDIR biology across BC and CRC. **A)** Venn diagram of differentially expressed genes between DDIR positive and DDIR negative in three cohorts shows nine common genes, including chemokines such as CCL5 and CXCL10. **B)** Ingenuity Pathway Analysis (IPA) was used to identify potential elevated/activated upstream regulators of the conserved 9 genes identified in (A). **C)** Correlation and distribution of DDIR compared to a sum cumulative score generated from the 9 gene overlap in (A). **D)** 15-month PFS (top) and 12-week objective response rate (bottom) comparing the Almac DDIR score and the modified 9-gene score. Estimates adjusted for WHO PS, left vs right-sided, liver resection, number of mets, source and age of sample, CMS, KRAS, BRAF, PIK3CA, TP53, MSI, imputed (N=361). **E)** Diagram displaying DDIR-positive and DDIR-negative specific tumour microenvironment and upregulation of biological features such as CXCL10 expression in CRC. DDIR-positive CRCs are riddled with immune infiltrates responding to inflammatory/interferon signalling leading to ‘inflamed’ TME. On the contrary, DDIR-negative CRCs are immune ‘cold’ with low level of CXCL10, interferon signalling and overall low immune cells.

Supplementary Figure S1

S1A Consort diagram for FOCUS trial samples



S1B Consort diagram for FOxTROT trial samples



Supplementary Figure S2

Figure S2a - Histogram of 391 patients with DDIR score in FOCUS trial (red line indicates 0.1094 threshold for positive DDIR classification)

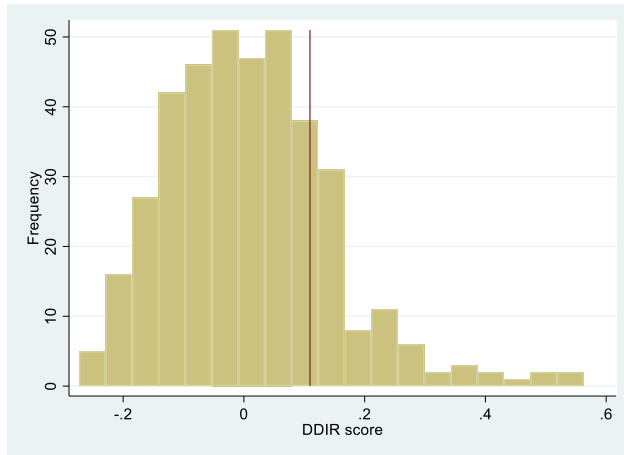


Figure S2b - Prognostic effect of DDIR status in metastatic colon cancer from the control arm of FOCUS

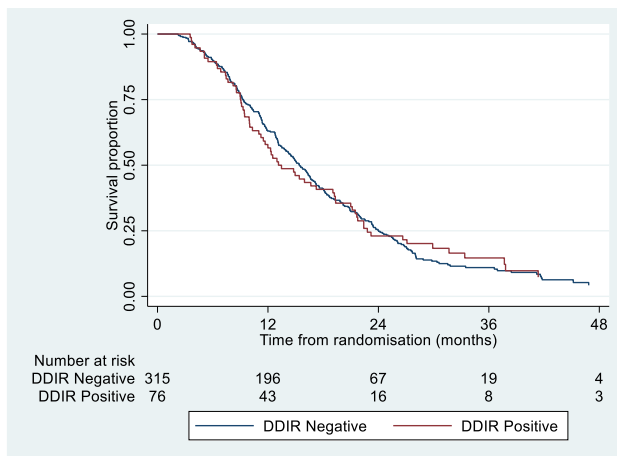


Figure S2c - Histogram of 93 patients with DDIR score in FOxTROT trial (red line indicates 0.1094 threshold for positive DDIR classification)

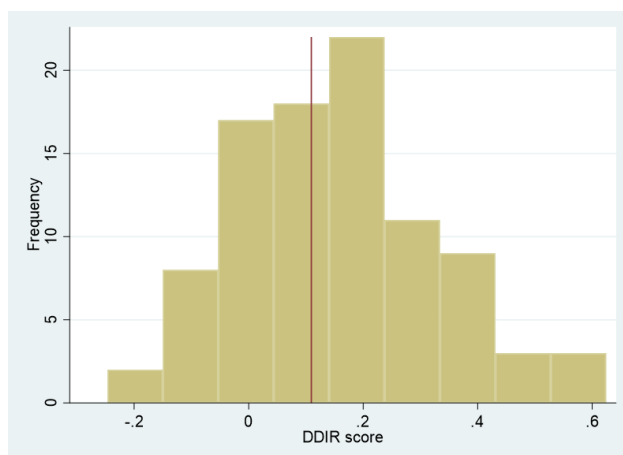


Table S1 - Baseline characteristics for FOCUS trial patients included in the DDIR analysis, broken down into 5FU alone versus 5FU+oxaliplatin groups, compared to the remaining FOCUS trial patients

* Rectal primaries excluded from DDIR analysis

† Patients may fall into multiple categories; totals may be >100%

Baseline characteristic	FOCUS patients included in DDIR analysis N=391					Remaining FOCUS Patients N=1744		P-value vs patients included in DDIR analysis
	5FU alone N=310		5FU + oxaliplatin N=81			N	%	
Mean (SD) age, years	64.0 (9.0)		61.8 (10.0)			62.3 (9.4)		0.019
	N	%	N	%		N	%	
Sex								
Male	196	63.2%	55	67.9%		1209	69.3%	0.049
Female	114	36.8%	26	32.1%		535	30.7%	
WHO performance status								
0	129	41.6%	34	42.0%		720	41.3%	0.27
1	164	52.9%	39	48.1%		869	49.8%	
2	17	5.5%	8	9.9%		155	8.9%	
Status of primary tumour at randomisation								
Resected	282	91.0%	69	85.2%		1163	66.7%	<0.001
Unresected/unresectable	18	5.8%	11	13.6%		505	29.0%	
Local recurrence	10	3.2%	1	1.2%		76	4.4%	
Site of primary tumour								
Colon	306	98.7%	78	96.3%		1013	58.1%	<0.001
Rectum *	0	0	0	0		711	40.1%	
Recto-sigmoid junction	1	0.3%	1	1.2%		5	0.3%	
Other	2	0.6%	2	2.5%		12	0.7%	
Missing	1	0.3%	0	0.0%		3	0.2%	
Location of metastases †								
Any metastases	307	99.0%	81	100.0%		1701	97.5%	0.037
Liver metastases	241	77.7%	65	80.2%		1307	74.9%	0.17
Liver-only metastases	87	28.1%	24	29.6%		507	29.1%	0.79
Nodal metastases	131	42.3%	33	40.7%		615	35.3%	0.013
Lung metastases	103	33.2%	25	30.9%		622	35.7%	0.27
Peritoneal metastases	46	14.8%	13	16.0%		229	13.1%	0.31
Other metastases	32	10.3%	16	19.8%		247	14.2%	0.33
Number of metastases								
0	3	1.0%	0	0.0%		43	2.5%	0.096
1	131	42.3%	30	37.0%		732	42.0%	
>1	176	56.8%	51	63.0%		969	55.6%	
Total	310	100%	81	100%		1744	100%	

Table S2 – Baseline characteristics of the biological sampled subset compared to all patients randomised to receive Pre and post operative FOLFOX in FOxTROT Trial

	Biological sample N=93	Total pre and post (n=698)
Mean age	67.4	63.0
SD	9.8	Range 27-83
Gender		
Male	55 (59%)	447 (64.0%)
Female	38 (41%)	251 (36%)
Tumour location		
Right sided	43 (46%)	340 (48.7%)
Left sided	46 (50%)	358 (51.3%)
pT stage¹		
pT0	0	4.1%
pT1/pT2	0	11.7%
pT3	(68) 73%	63.7%
pT4	(23) 25%	20.5%
pN stage¹		
N0	21 (22.6%)	59.4%
N1	44 (47%)	25.4%
N2	28 (30%)	15.2%
MSI status		
MSI	3 (3%)	173 (25%)
MSS	88 (95%)	592 (85%)
RAS status		
wildtype	73 (83%)	302 (63%) ²
mutant	15 (17%)	180 (37%) ²
Not tested		216 (30.9%)

¹ Pathological staging performed according to TNM version 5

² as proportion of all samples tested

Supplementary Figure 3

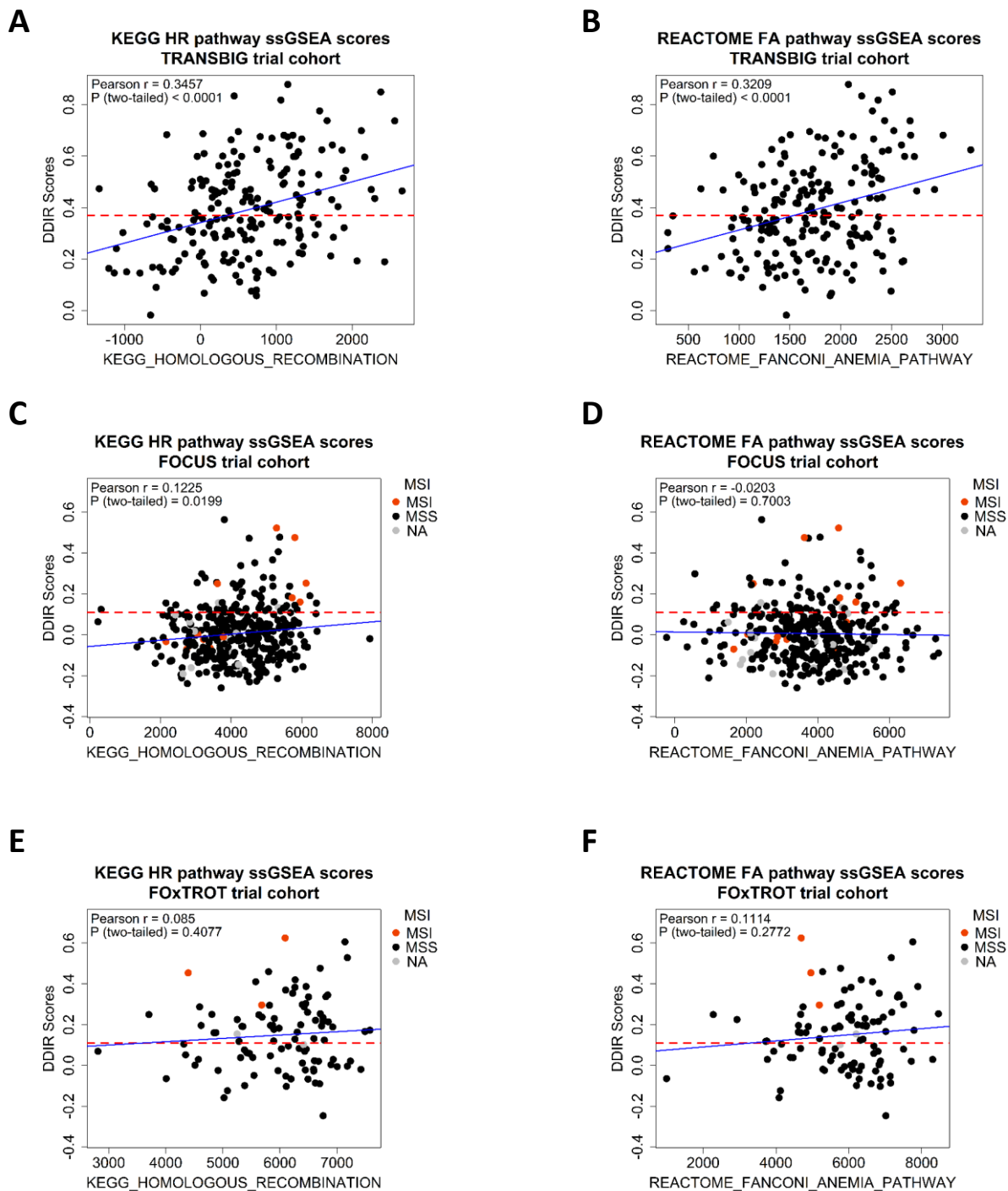


Figure S3. Association of DNA damage response and repair pathways with DDIR subtypes vary between breast (BC) and colorectal cancer (CRC). **A**) Correlation between DDIR continuous scores and single sample gene set enrichment analysis (ssGSEA) scores for TRANSBIG BC cohort on KEGG Homologous Recombination and **B**) REACTOME Fanconi Anemia pathway, with line of best of fit (blue) and DDIR threshold value indicated with red dash line at 0.37 for BC. **C**) Correlation of KEGG Homologous Recombination and **D**) REACTOME Fanconi Anemia pathway ssGSEA scores with DDIR scores in FOCUS CRC cohort, DDIR threshold indicated with red dash line at 0.1094 for CRC along with MSI status (MSI = red, MSS = black, NA = grey). **E**) Correlation of KEGG Homologous Recombination and **F**) REACTOME Fanconi Anemia pathway ssGSEA scores with DDIR scores in FOXOTROT CRC cohort, DDIR threshold indicated with red dash line at 0.1094 for CRC along with MSI status (MSI = red, MSS = black, NA = grey).

Supplementary Figure 4

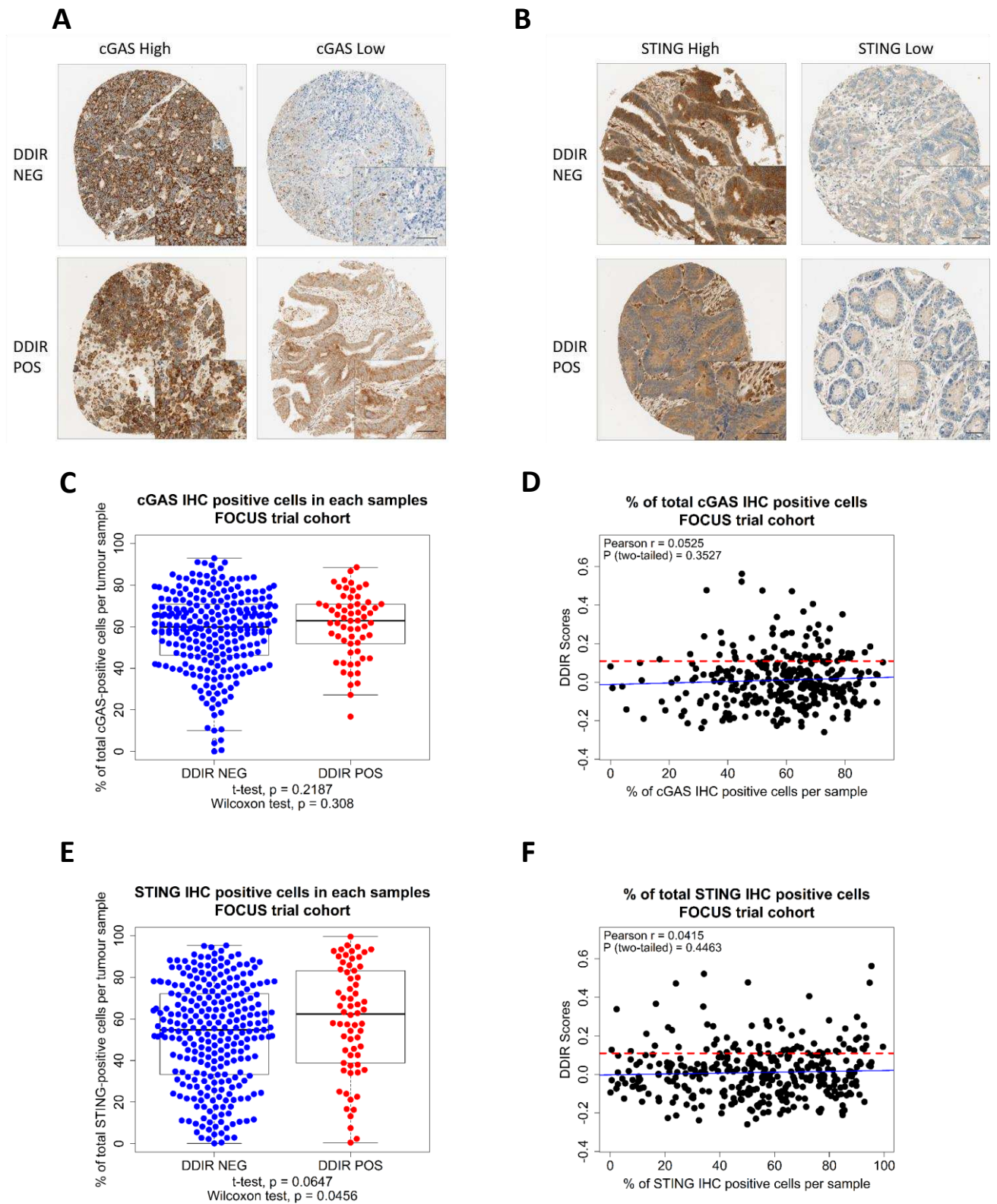
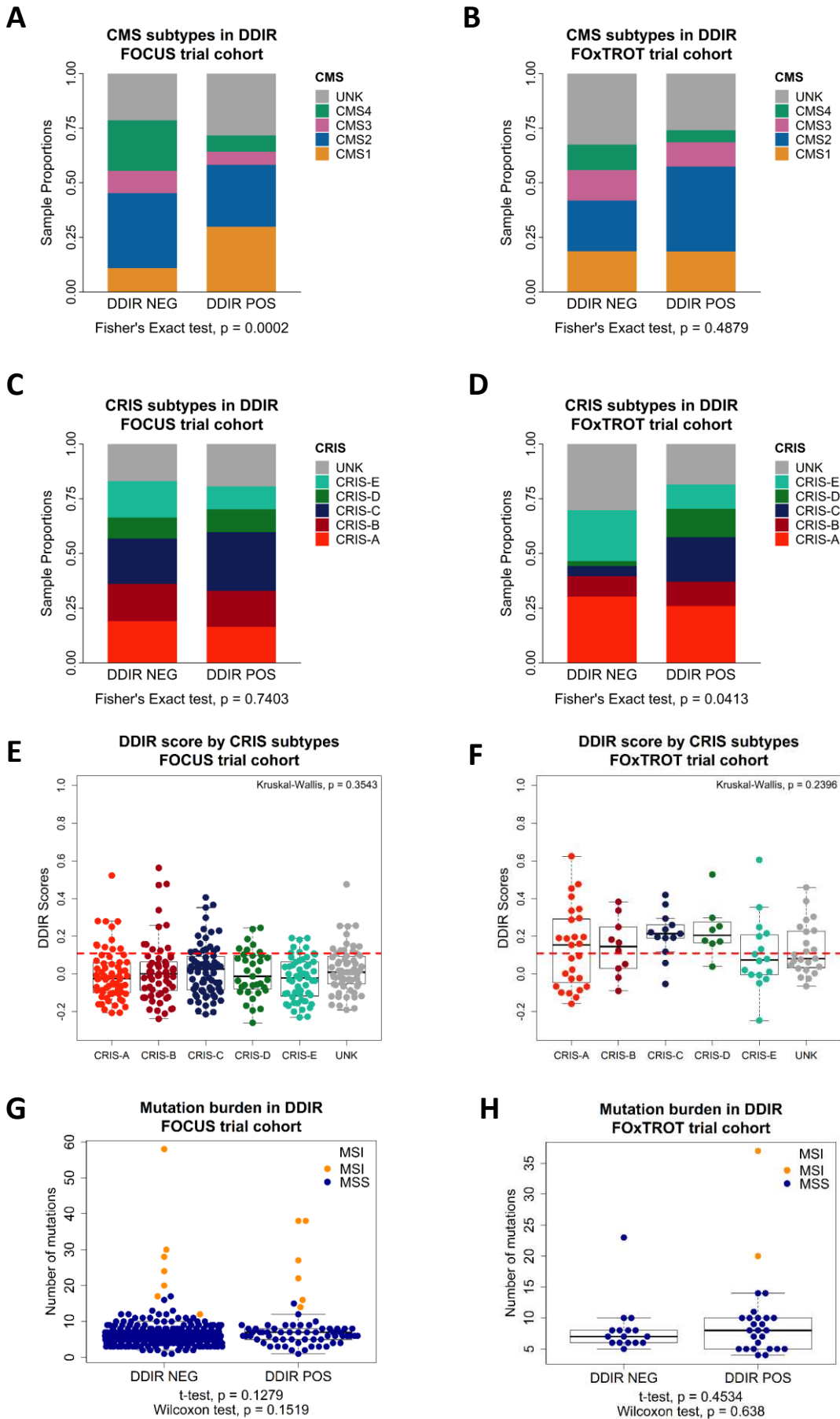


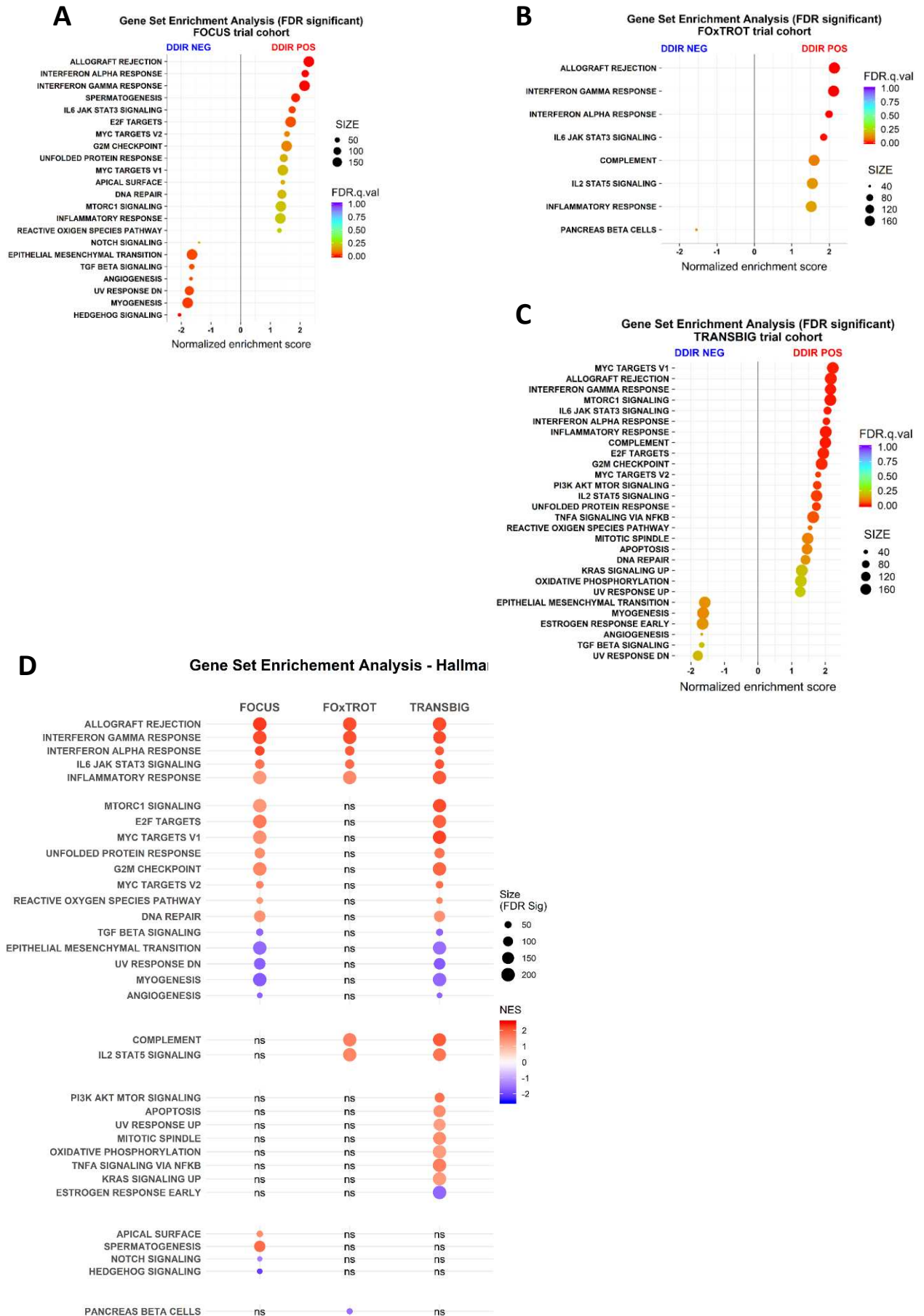
Figure S4. Expression of cGAS and STING reveals lack of association between innate immune response and DDIR positivity in colorectal cancer. **A**) Immunohistochemistry images of DDIR positive and DDIR negative tumours stained with cGAS and **B**) STING, (x10; inset x20, 50µm bar). **C**) Percentage of total cells in tumour samples positively stained with cGAS comparing DDIR negative and DDIR positive tumours in boxplot, and **D**) Correlation between percentage of total cGAS-positive cells and DDIR scores, shown with line of best fit (blue) and DDIR threshold at 0.1094 (red dash line). **E**) Percentage of total cells in tumour samples positively stained with STING comparing DDIR negative and DDIR positive tumours in boxplot, and **F**) Correlation between percentage of total STING-positive cells and DDIR scores, shown with line of best fit (blue) and DDIR threshold at 0.1094 (red dash line). Statistics: Student's *t*-test, Wilcoxon rank sum test and Pearson's Coefficient Correlation.

Supplementary Figure 5



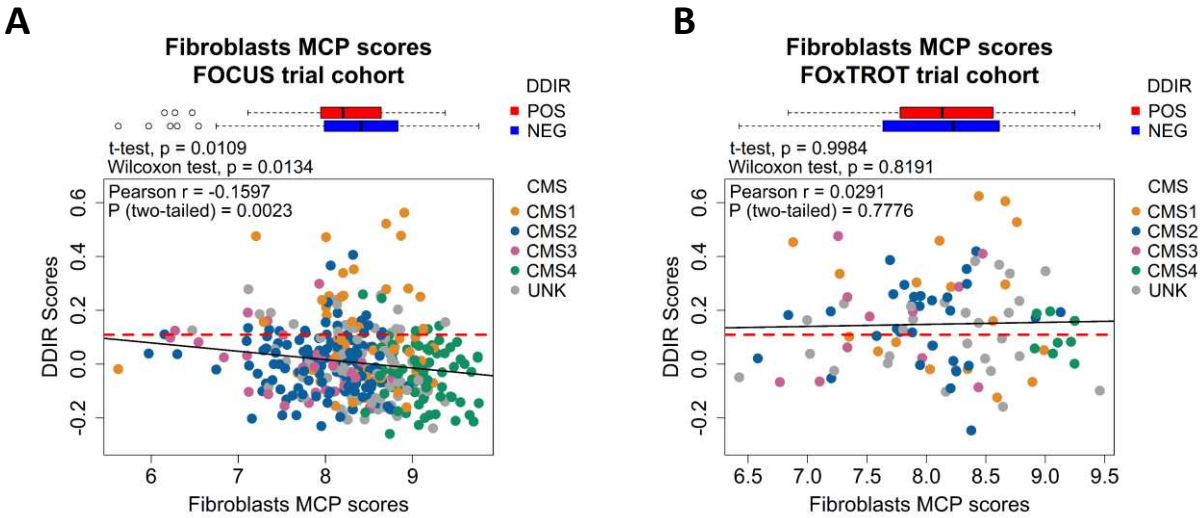
Supplementary Figure S5. CMS1 samples show enrichment in DDIR-positive tumours, while displaying no association of CRIS with DDIR. **A)** Proportion of CMS samples in DDIR positive and DDIR negative shown for FOCUS and **B)** FOxTROT cohort. **C)** Proportion of CRIS samples in DDIR positive and DDIR negative shown for FOCUS and **D)** FOxTROT cohort. Statistics: Fisher's exact test. **E)** Distribution of CRIS samples against DDIR score in FOCUS and **F)** FOxTROT cohort, shown with DDIR threshold value at 0.1094 (red dash line). Statistics: Kruskal-Wallis rank sum test for global p -value. **G)** Boxplot depicting comparison of mutational burden in DDIR positive and DDIR negative tumours in FOCUS cohort, with overlay of MSI status. **H)** Comparison of mutational burden in DDIR positive and DDIR negative tumours in FOxTROT cohort. Statistics: Pearson's Coefficient Correlation, Student's t -test and Wilcoxon rank sum test.

Supplementary Figure 6



Supplementary Figure S6. Gene set enrichment analysis for FOCUS, FOxTROT and TRANSBIG cohorts. **A)** Dot plot of GSEA between DDIR negative (left panel) and DDIR positive (right panel) tumours with FDR significant (<25%) gene sets and size indicating number of genes in the gene set for FOCUS, **B)** FOxTROT and **C)** TRANSBIG cohort. **D)** Dot plot with significant gene sets identified in at least one or more cohorts (FOCUS, FOxTROT or TRANSBIG) indicated with dots and non-significant gene set as 'ns'; the normalised enrichment score (NES) indicates the enrichment of gene set in DDIR positive (red) or DDIR negative (blue) tumours.

Supplementary Figure 7



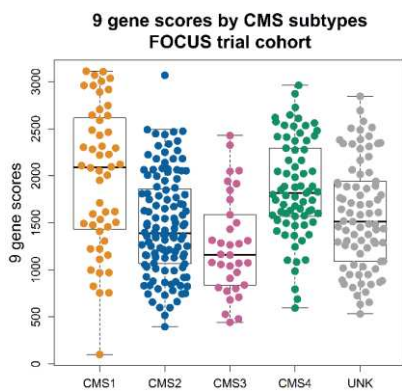
Supplementary Figure S7. Contribution of stromal fibroblast infiltrates in DDIR negative tumours. **A)** Comparison of fibroblast MCP estimates between DDIR positive (red) and DDIR negative (blue) tumours shown in boxplot above scatterplot examining correlation between DDIR continuous score and fibroblast MCP score, with overlay of CMS samples in FOCUS and **B)** FOxTROT cohort.; line of best fit in black, DDIR threshold value at 0.1094 (red dash line). Statistics: Student's t -test, Wilcoxon rank sum test and Pearson's Coefficient Correlation.

Supplementary Figure 8

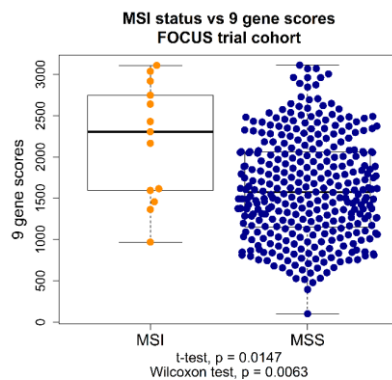
A

Upstream Regulator	Molecule Type	Activation z-score	Target Molecules in Dataset
IFNG	cytokine	2.811	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2,UBD
IFNA2	cytokine	2.776	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2,UBD
TLR7	transmembrane receptor	2.63	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2
Interferon alpha	group	2.603	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2,SLAMF7
IL1B	cytokine	2.595	CCL5,CXCL10,CXCL9,IDO1,IFIT1,OAS2,UBD
IRF7	transcription regulator	2.568	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2
TNF	cytokine	2.408	CCL5,CXCL10,CXCL9,IDO1,IFIT1,OAS2,UBD
STAT1	transcription regulator	2.403	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2,UBD
TLR9	transmembrane receptor	2.397	CCL5,CXCL10,CXCL9,IDO1,IFI44L,IFIT1,OAS2
IFN Beta	group	2.234	CCL5,CXCL10,IDO1,IFIT1,OAS2
IFNB1	cytokine	2.23	CCL5,CXCL10,CXCL9,IDO1,IFIT1,OAS2
IFNL1	cytokine	2.229	CXCL10,CXCL9,IFI44L,IFIT1,OAS2
NFkB (complex)	complex	2.187	CCL5,CXCL10,CXCL9,IDO1,UBD
RELA	transcription regulator	2.187	CCL5,CXCL10,CXCL9,OAS2,UBD
PRL	cytokine	2.176	CXCL10,CXCL9,IFI44L,IFIT1,OAS2
RNY3	other	2	CXCL10,IFI44L,IFIT1,OAS2
PAF1	other	2	CCL5,IDO1,IFI44L,OAS2
TGM2	enzyme	2	CXCL10,IFIT1,OAS2,SLAMF7

B



C



Supplementary Figure S8. Contribution of stromal fibroblast infiltrates in DDIR-negative tumours. **A)** Ingenuity pathway analysis (IPA) was used to identify potential elevated/activated upstream regulators of the conserved 9 genes **B)** Relationship of 9-gene score to CMS classification in the FOCUS cohort. **C)** Relationship of 9-gene score to MSI classification in the FOCUS cohort.