Immune Activation by DNA Damage Predicts Response to Chemotherapy and Survival in Oesophageal Adenocarcinoma

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Running title: DDIR assay is predictive of survival in oesophageal adenocarcinoma

This work is funded in part by:

Belfast Health and Social Care Trust Gastrointestinal Cancer Research Fund, Almac Diagnostics, Medical Research Council, Cancer Research UK, HSC Research and Development Division of the Public Health Agency in Northern Ireland, National Institute

for Health Research (NIHR) Cambridge Biomedical Research Centre, Invest Northern Ireland

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Abstract Word Count: 250
Article Word Count: 4575
Number of Figures: 3; Number of Tables: 3
Number of Supplementary Figures: 5
Number of Supplementary Tables: 10

Keywords: Oesophageal Cancer, Predictive, Biomarker, Gene Expression

Abbreviations:

OAC - Oesophageal Adenocarcinoma

DDIR - DNA Damage Immune Response

OCCAMS - Oesophageal Cancer Clinical and Molecular Stratification Consortium

PD-L1 - Programmed Death Ligand 1

FFPE – Formalin Fixed Paraffin Embedded

cGAS - cyclic GMP-AMP synthase

STING - Stimulator of interferon genes

Abstract

Objective

Current strategies to guide selection of neo-adjuvant therapy in oesophageal adenocarcinoma (OAC) are inadequate. We assessed the ability of a DNA Damage Immune Response (DDIR) assay to predict response following neo-adjuvant chemotherapy in OAC.

Design

Transcriptional profiling of 273 formalin fixed paraffin embedded (FFPE) pre-chemotherapy endoscopic OAC biopsies was performed. All patients were treated with platinum-based neo-adjuvant chemotherapy and resection between 2003 and 2014 at four centres in the OCCAMS consortium. CD8 and Programmed Death Ligand 1 (PD-L1) immunohistochemical staining was assessed in matched resection specimens from 126 cases. Kaplan-Meier and Cox Proportional Hazards regression analysis were applied according to DDIR status for recurrence-free (RFS) and overall survival (OS).

Results

A total of 66 OAC samples (24%) were DDIR positive with the remaining 207 samples (76%) being DDIR negative. DDIR assay positivity was associated with improved RFS (HR 0.61; 95% CI 0.38-0.98; p=0.042) and OS (HR 0.52; 95% CI 0.31-0.88; p= 0.015) following multivariate analysis. DDIR positive patients had a higher pathological response rate (p= 0.033), lower nodal burden (p= 0.026) and reduced circumferential margin involvement (p= 0.007). No difference in OS was observed according to DDIR status in an independent surgery-alone dataset.

DDIR positive OAC tumours were also associated with the presence of CD8+ lymphocytes (intra-tumoural $p < 0.001$; stromal $p = 0.026$) as well as PD-L1 expression (intra-tumoural $p = 0.047$; stromal $p = 0.025$).

Conclusion

The DDIR assay is strongly predictive of benefit from DNA-damaging neo-adjuvant chemotherapy followed by surgical resection and is associated with a pro-inflammatory micro-environment in OAC.

Significance of this study

What is already known about this subject?

- Neo-adjuvant therapy followed by surgical resection cures less than half of patients with resectable oesophageal adenocarcinoma (OAC).
- Response rates to neo-adjuvant platinum-based chemotherapy are low at 15%.
- Recent molecular landscape studies in OAC have indicated the presence of a DNA Damage Response impaired subgroup of tumours.

What are the new findings?

- A 44 gene DNA Damage Immune Response (DDIR) assay can successfully be applied to FFPE pre-treatment endoscopic biopsies with a success rate of >98%.
- The DDIR assay is predictive of response and survival benefit following DNA-damaging neo-adjuvant chemotherapy and surgery.
- DDIR positive patients have increased pathological response, lower nodal burden and reduced resection margin involvement.
- DDIR positivity is associated with an inflammatory micro-environment characterised by the presence of CD8 positive Tumour Infiltrating Lymphocytes and high PD-L1 expression.

How might it impact on clinical practice in the foreseeable future?

- The ability to select the appropriate neo-adjuvant therapy for individual OAC patients could increase pathological response rates and survival.
- Ineffective therapy could be avoided in OAC patients unlikely to respond.
- Insights into the molecular biology of the DDIR subgroup will allow novel combinations of conventional therapy with DNA repair inhibitors or immunotherapy to be explored.

INTRODUCTION

The incidence of OAC in the Western world has risen 6-fold in the last forty years with the highest incidence occurring in the UK.[1–3] In resectable cases the addition of neo-adjuvant or peri-operative therapy provides a modest improvement in overall survival but only 15% of patients demonstrate a histopathological response to therapy in the resected tumour.[4–7] Despite improvements in oncological and surgical management the majority of patients relapse and die of their cancer.[4–6] Therefore, there is a pressing need to identify biomarkers capable of predicting response in order to select the appropriate neo-adjuvant therapy for individual patients.

Imaging and molecular features of OAC have been studied in an attempt to identify predictive biomarkers to neo-adjuvant therapy. For example, serial [^{18}F]-2-fluoro-2-deoxy-d-glucose (18FDG) Positron Emission Tomography (FDG-PET) scans can detect changes in tumour metabolism with the aim of predicting pathological response.[8–10] A 35% reduction in Standard Uptake Value (SUV) 14 days after baseline has been correlated with a higher rate of tumour regression, R0 resection and improved survival in a prospective study of resectable OAC.[9] However, 42% of FDG-PET responders identified by a reduction in SUV did not in fact achieve a pathological response highlighting the pressing need to identify more accurate molecular predictive biomarkers. Various proposed single gene predictive biomarkers, such as Nuclear-Factor-κβ, Epidermal Growth Factor Receptor (EGFR), TP53, ERCC1 and Thymidylate Synthase (TS), have met with limited success as they fail to capture the complex biology of OAC.[11–18] Recent advances in the molecular understanding of OAC have demonstrated that it is a disease characterized by a high level of mutations and copy number changes giving rise to prominent intra-tumoural heterogeneity.[19–22] To encapsulate the biology underpinning response to chemotherapy in OAC a number of studies have applied gene expression profiling to pre-treatment endoscopic biopsies to identify a predictive gene signature.[23–25] However, these

signatures rely on fresh frozen tissue, which is not routinely available, and have been developed in small discovery cohorts without independent validation.

The DNA Damage Immune Response (DDIR) assay, formerly known as the DNA Damage Response Deficiency (DDRD) assay, was previously developed in breast cancer using an unsupervised hierarchical clustering approach.[26] When tested in an independent breast cancer dataset (n=203) DDIR-positivity was associated with an odds ratio (OR) for pathological response following neo-adjuvant chemotherapy of 3.96 (95% CI 1.67-9.41; p= 0.002) and in a cohort of 191 node-negative breast cancer patients the assay predicted 5 year disease-free survival (DFS) following adjuvant chemotherapy with a hazard ratio (HR) of 0.37 (95% CI 0.15-0.88; p=0.025). Further validation in 664 chemo-naive patients indicated that the DDIR assay was not prognostic and only predicts outcome in the context of DNA-damaging chemotherapy. Biologically the DDIR assay indicates constitutive activation of the cyclic GMP-AMP synthase (cGAS)/Stimulator of interferon genes (STING) pathway in response to endogenous DNA damage.[27] Deficiencies in DNA repair and the Fanconi Anaemia/BRCA pathway in particular, have been reported to activate this pathway. Importantly the 44 gene DDIR assay includes well known immune checkpoint targets, such as PD-L1 and Indoleamine 2,3-Dioxygenase 1 (IDO-1), as well as several inflammatory cytokines. Immune activation via the STING pathway results in infiltration of the tumour by T lymphocytes and upregulation of immune checkpoints to create an inflammatory micro-environment associated with chemo-sensitivity. However, pathological response (TRG 1/2) to DNA-damaging chemotherapy and chemo-radiotherapy occurs in only 15% and 23% of OAC tumours, respectively.[6,7] We hypothesized that pathological tumour response and improved survival may be due to pre-existing deficiencies in DNA repair pathways with associated activation of an innate immune response. An assay which could identify this subgroup of OAC tumours would predict benefit from neo-adjuvant chemotherapy.

We, therefore, assessed the ability of the DDIR assay to predict pathological response and prognosis following DNA-damaging neo-adjuvant chemotherapy in OAC. We demonstrate that the DDIR assay can be applied to routine diagnostic clinical specimens to allow the selection of patients for whom DNA-damaging chemotherapy would be beneficial. DDIR positivity is also strongly correlated with the presence of tumour infiltrating lymphocytes (TILs) and PD-L1 expression indicating an association between deficiencies in DNA damage repair mechanisms and a pro-inflammatory micro-environment in OAC.

MATERIALS AND METHODS

This study was performed according to the REporting recommendations for tumour MARKer prognostic studies (REMARK) as outlined in the criteria checklist (Supplementary Table 1) and REMARK study design diagram (Supplementary Figure 1, Appendix A)

Patient Samples

FFPE pre-chemotherapy endoscopic biopsies from 273 patients with resectable OAC, treated with neo-adjuvant chemotherapy followed by surgical resection, were collected at four UK centres in the Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) consortium between 2003 and 2014 (Supplementary Table 2). Follow up was performed according to local institutional guidelines. Patients with localized histologically confirmed adenocarcinoma of the oesophagus or gastro-oesophageal junction were included and all patients were followed up for at least two years. Pathological response was assessed in the matched resection specimens according to the method described by Mandard et al with a responder defined as Tumour Regression Grade (TRG) ≤ 2.[7,28] Assuming a marker positive rate of 21% (estimated from preliminary data) a sample set of 273 patients had an 80% power to detect a Hazard Ratio (HR) of 2. Relevant ethical approvals were obtained from the Northern Ireland Biobank (NIB12-0032) and the Office for Research Ethics Committees Northern Ireland (ORECNI, 13/NI/0149).

For independent *in silico* validation a publically available dataset of 57 OAC resections which did not receive DNA-damaging chemotherapy (GSE19417) was assessed (Supplementary Table 3). All tumour samples were collected and snap-frozen from patients undergoing potentially curative surgical resection at the Bristol Royal Infirmary between 1992 and 2000. Gene expression profiling was performed using a custom-made Agilent 44K 60-mer oligo-microarray as previously described.[29]

Gene Expression Profiling from FFPE Tissue

Biopsies were reviewed for pathological subtype prior to marking for macrodissection and samples containing at least 50% adenocarcinoma tissue by area were taken forward. Where tumour material was limited endoscopic biopsy fragments from the same patient were pooled. Total RNA was extracted using the Recoverall™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific, Waltham, MA) and amplified using the NuGen Ovation FFPE Amplification System v3 (NuGen San Carlos, CA). The amplified product was hybridized to the Almac Diagnostics Xcel™ array (Almac, Craigavon, United Kingdom), a cDNA microarray-based technology optimized for archival FFPE tissue, and analysed using the Affymetrix Genechip® 7G scanner (Affymetrix, Santa Clara, CA) as previously described.[30,31] Functional enrichment was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Raw expression data is available at the Array Express repository (Accession Number E-MTAB-6969).

Immunohistochemistry (IHC)

Matched FFPE OAC resection specimens were available for 126 patients who received neo-adjuvant chemotherapy prior to surgical resection at the Northern Ireland Cancer Centre. Pathological staging was defined according to International Union Against Cancer (UICC) TNM staging, 7th edition and the cases had a median follow up time of 48.8 months (Supplementary Table 4). All cases were represented in triplicate and the TMAs were constructed as previously described.[32,33]

Antibodies to CD8 (C8/144B, M7103, Dako) and PD-L1 (SP142, Roche) were used as previously described.[27] Tissue microarray sections were scored by two independent observers (EP & EMcC) who were blinded to the clinical data. A semiquantitative scoring

system was used for CD8+ expression with a score of 3 indicating strong CD8+ expression, 2 moderate expression, 1 weak expression and 0 absence of expression. For PD-L1, tumour and stroma were scored for percentage of cells with positive expression and previously published cut-offs of 1% or greater and 5% or greater were used for analysis.[34]

Whole Genome Sequencing

Matched whole genome sequencing data was available for 44 patients who received neo-adjuvant chemotherapy prior to surgical resection at three OCCAMS centres (Cambridge, Edinburgh and Southampton; Supplementary Table 4).

Whole genome sequencing was performed and mutational signatures identified using the non-negative matrix factorization (NMF) methodology as previously described.[22,35]

Statistical Analysis

Microarray data was pre-processed using the Robust Multi-array Average (RMA) model for the Almac Diagnostics Xcel™ array with DDIR signature scores calculated and pre-defined cut-points applied as previously described.[30] A threshold of 0.3403 was optimised in an independent technical study of n= 45 OAC samples and applied independently to the validation cohort dichotomising patients as DDIR positive (>0.3403) or DDIR negative (≤0.3403). Cox proportional hazards regression was used to investigate the prognostic effects of the DDIR signature on relapse-free (RFS) and overall survival (OS) defined as the time from surgical resection to relapse of disease or death from any cause, respectively. The estimated effect of the signature was adjusted for factors available at the time of diagnosis (clinical tumour status, clinical nodal status and tumour grade) by fitting a multivariate model.

Further details are available in the Supplementary Methods.

RESULTS

Assessment of the DDIR assay and survival following neo-adjuvant chemotherapy and surgical resection in OAC

To assess the ability of the DDIR assay to predict survival following neo-adjuvant DNA-damaging chemotherapy and resection in OAC it was applied to a retrospective dataset of 273 FFPE biopsy samples. A total of 66 OAC samples (24%) were characterized as DDIR positive with the remaining 207 (76%) being DDIR negative. Significantly lower rates of lymph node and circumferential resection margin (CRM) involvement a more proximal tumour location and older age were observed for DDIR positive tumours (Table 1). DDIR assay positivity was associated with improved RFS (HR 0.58, 95%CI 0.38-0.90; p= 0.015) and OS (HR 0.62, 95%CI 0.41-0.95; p= 0.029) following surgical resection (Figure 1). When evaluated as a continuous variable, higher DDIR scores were associated with both improved RFS (HR 0.34, 95% CI 0.13-0.93; p=0.036) and OS (HR 0.32, 95% CI 0.12-0.87; p=0.026). Univariate analysis confirmed associations between survival and pre-surgical clinical N stage as well as post-surgical factors such as pathological T and N stage, differentiation, lymphovascular invasion and circumferential resection margin status (Supplementary Table 5). Applying a published cut off of ≥15 lymph nodes to indicate an adequate lymph node yield we found that there was no association between the DDIR status and lymph node yield and neither was there an association between adequate lymph node yield and relapse-free (HR 0.94, 95% CI 0.66-1.39; p= 0.847) or overall survival (HR 1.02, 95% CI 0.7-1.48; p= 0.916). Whilst the Lauren classification is known to be prognostic in OAC it was not available for the whole cohort and so it is unclear how the DDIR assay relates to intestinal versus diffuse type adenocarcinomas.[36] Also, there was no association between the administration of post-operative chemotherapy and DDIR status (HR 0.74, 95% CI 0.39-1.4; p= 0.354) but there was a trend towards improved overall survival in DDIR positive patients when no adjuvant chemotherapy was administered (HR 0.39, 95% CI 0.15-1.02; p= 0.55) (Supplementary Figure 2).

Multivariable analysis was performed to test the association between DDIR status and each survival endpoint following adjustment for factors available at diagnosis (Table 2). DDIR positive patients had improved RFS relative to DDIR negative patients (HR 0.61, 95%CI 0.38-0.98; p= 0.042) and assay positivity was also independently associated with improved OS (HR 0.52, 95%CI 0.31-0.88; p= 0.015).

To assess whether the DDIR assay was prognostic, independent of DNA-damaging chemotherapy treatment, it was applied to a publically available dataset of 57 OAC resections which did not receive neo-adjuvant chemotherapy (Supplementary Table 3). No significant difference in overall survival was noted between the DDIR positive and DDIR negative populations (HR 0.86, 95%CI 0.48-1.55; p= 0.61) (Supplementary Figure 3). However, further confirmatory results in a larger cohort are required. Taken together these results indicate that the DDIR assay is a strong predictor of survival benefit following surgical resection in OAC, but only in the context of neo-adjuvant DNA-damaging chemotherapy.

The DDIR assay is predictive of pathological response in OAC

The ability to predict pathological response to neo-adjuvant chemotherapy would improve patient stratification and treatment selection in OAC. TRG was available for 228 patients in the OAC cohort with 24 (11%) of cases having a TRG $\leq$ 2, indicating a pathological response, and 203 (89%) TRG 3-5, in keeping with limited or no response to chemotherapy. Pathological response was observed in 16.7% and 6.8% of DDIR positive and DDIR negative cases respectively (p= 0.025) (Table 1). DDIR scores were grouped by response status and one-way ANOVA analysis demonstrated significantly higher DDIR scores in responders compared to non-responders (p= 0.033). This indicates that the DDIR score was significantly enriched for tumours that respond to neo-adjuvant chemotherapy in OAC (Figure 2).

DDIR assay positivity and Tumour Mutational Load

Recent sequencing studies have stratified OAC into subtypes defined by the pattern of somatic mutations. Secrier et al identified three subgroups (C>A/T Dominant, DNA Damage Response (DDR) Impaired and Mutagenic through the application of mutational signatures to WGS data from a cohort of 129 chemotherapy-naïve OAC samples.[37] We sought to assess the overlap between cases defined as DDIR positive by our gene expression assay and DDR impaired by mutational signature analysis. A total of 44 cases had matched gene expression and WGS data available and demonstrated higher clinical nodal staging and different neo-adjuvant chemotherapy regimens compared to the whole cohort and the TMA subset (Supplementary Table 4). This may reflect differing staging methodologies used at the largest contributing centre to the WGS cohort (University of Cambridge; 29 (66%) of patients) and the increased use of cisplatin and oxaliplatin doublet neo-adjuvant regimens due to clinical trials recruiting at that centre at the time of sample collection (OEO5[38], LEO[39]).

Non-negative matrix factorization (NMF) was applied to cluster the patients into the three subgroups (Supplementary Figure 4). No association was observed between the DDIR status and the predominant mutational signature (Supplementary Table 6; p= 0.83). Although the size of the cohort limits the statistical power of the analysis, DDIR positive patients did display a trend towards higher tumour mutational burden and a higher mutation rate (Supplementary Figure 5). However, no significant differences were observed in the mean copy number or total number of deleterious somatic mutations or indels in multiple DDR pathways between the DDIR positive and negative samples. Neither were there any differences observed in the copy number of genes involved in the homologous recombination, double and single strand break repair pathways (data not shown). Whilst both assays are related to loss of DNA repair the assessment of differing

biologies represented by immune activation in response to DNA damage measured by the DDIR assay, as opposed to the pattern of mutations caused by deficiencies in DNA repair mechanisms, may lead to the lack of association.

DDIR assay positivity is associated with CD8+ T-Lymphocytes and Expression of PD-L1

We hypothesized that increased DNA damage in DDIR positive tumours may be associated with increased lymphocytic infiltration and upregulation of immune checkpoint genes. A list of 45 genes differentially expressed between DDIR positive and negative patients, with a fold change of >2, was generated (Supplementary Table 7). As expected, this list included the genes from the DDIR signature, with 5 out of 44 genes represented, but it also included a number of genes encoding inflammatory cytokines and mediators of an immune response. Chemokines such as CXCL9 and CXCL13 showed 5.5 and 4.58 fold upregulation respectively and 29 of the 45 genes (64.4%) have a role in the immune response. Pathway analysis demonstrated enrichment of a wide range of biological processes related to immune activation and viral response (Supplementary Table 8), further strengthening the association of DDIR positive status with a pro-inflammatory micro-environment.

To assess the relationship between DDIR status, PD-L1 expression and the presence of tumour-infiltrating lymphocytes (TILs) we performed IHC analysis on 126 resection specimens matched to patients in the gene expression cohort (Figure 3, Table 3, Supplementary Tables 9 &10). Previously published cut-offs of 1% or greater and 5% or greater were used to define PD-L1 positivity. A statistically significant association was observed between DDIR assay positivity and intra-tumoural and stromal PD-L1 expression at the 5% cut-off ($p= 0.047$; $p= 0.25$, respectively). The presence of both intra-tumoural

and stromal CD8+ TILs was also associated with DDIR assay positivity (p, 0.001; p= 0.026, respectively).

DISCUSSION

We have demonstrated that the DDIR assay is predictive of response and independently prognostic following DNA-damaging neo-adjuvant chemotherapy and surgical resection in OAC. DDIR assay positivity was associated with improved survival following chemotherapy and surgery and identified those patients with a higher probability of obtaining a pathological response, reduced nodal burden and clear resection margins. When assessed alongside clinical factors available at the time of diagnosis DDIR status demonstrated superior prognostic ability compared to standard clinicopathological factors. Application of the DDIR assay to a cohort of patients who did not receive neo-adjuvant therapy demonstrated no difference in survival according to DDIR status indicating that the DDIR assay may not be prognostic in its own right but only in the context of DNA-damaging therapy.

Our study has a number of advantages compared to prior attempts to identify a predictive biomarker to neo-adjuvant therapy in OAC. Previous biomarker studies have relied upon fresh frozen tissue, which is not routinely collected, and suffered from high attrition rates for samples analysis. However, our study utilised FFPE diagnostic tissue with a success rate of 95.8% in samples submitted for analysis, allowing the assay to be readily applied to clinical practice. Other attempts to develop a predictive classifier have also been limited by small sample size and lack of suitable validation sets.[23–25,40] We were able to validate the DDIR assay in a sufficiently powered real-world cohort of patients to assess its predictive ability and the assay has also undergone extensive analytical validation enabling it to be reproducibly applied to clinical samples.

Limitations of the study include the use of a retrospective clinical cohort which may influence survival outcomes due to the absence of standardised follow-up procedures and so the DDIR assay will require further validation in a randomised controlled trial dataset and by a prospective study. Also, all patients were treated with neo-adjuvant platinum-

based chemotherapy prior to surgical resection. Considering neo-adjuvant chemo-radiotherapy is standard practice in the US and many parts of Europe further validation is required in a sample set treated with this modality. An additional challenge for many biomarker studies is the heterogeneity demonstrated by OAC. A high level of intra-tumoural heterogeneity has been correlated with response to neo-adjuvant chemotherapy in OAC and has indicated the limitations of a single biopsy to develop a predictive biomarker.[41] This may be partially mitigated in our study by the pooling of endoscopic biopsy fragments with sufficient tumour material but only samples from multiple sites within the tumour could encompass the underlying clonality of OAC tumours. The limited amount of tumour tissue available in the biopsy samples also precluded their use in the analysis of TILs and PD-L1 expression and so matched resection specimens were used. However, the prior administration of neo-adjuvant chemotherapy may have influenced the amount of TILs present and the expression levels of PD-L1 in these specimens.

With regard to the clinical applicability of the assay a number of factors should be taken into consideration. The response rate of 16.7% observed in DDIR positive patients was significantly higher than that observed in DDIR negative patients (6.8%) but is comparable to unselected published retrospective and clinical trial cohorts.[4,7] This may limit the utility of the assay as a tool to enhance pathological response following neo-adjuvant chemotherapy. Conversely, a response rate of 6.8% in DDIR negative patients may not be low enough to dissuade clinicians from using neo-adjuvant cisplatin-based chemotherapy in this patient population. Data from other cancer types indicating an increase in response following taxane treatment in tumours with intact DNA repair mechanisms may provide a rationale for the use of the docetaxel, oxaliplatin, fluorouracil/leucovorin (FLOT) chemotherapy regimen in DDIR negative patients. For example, ovarian cancer patients with low/intermediate levels of BRCA1 have improved survival following treatment with platinum-based chemotherapy whereas high levels of BRCA1 expression correlate with improved overall survival following the use of taxane-

contatining chemotherapy.[42] Similarly, in breast cancer cell lines exogenous expression of BRCA1 increased sensitivity to spindle poisons, such as paclitaxel and vinorelbine.[43] We would hypothesise that DDIR positive patients may benefit from the direct damage to DNA induced by cisplatin or radiotherapy, whereas the DDIR negative cases may also require the addition of inhibitors of microtubule formation, such as docetaxel. Testing of the assay in sufficiently powered randomised trial cohorts containing suitable treatment regimens could answer such a question. Further considerations regarding the utility of the assay include the association of DDIR positivity with older patients which could indicate an increased prevalence of this pro-inflammatory subgroup with increasing age. Also, the trend towards increased survival for DDIR positive patients who do not receive adjuvant chemotherapy should be interpreted with caution as it is likely to be confounded by patients who had an excellent pathological response not going on to receive further chemotherapy.

The biology of a DNA repair deficient subgroup should be examined in the context of recent publications from collaborative sequencing efforts which have characterized the molecular landscape of oesophago-gastric adenocarcinoma.[19,20,44,45] Multiple platform analysis by The Cancer Genome Atlas (TCGA) has identified four subgroups within oesophago-gastric adenocarcinoma with tumours of the distal oesophagus and gastro-oesophageal junction characterized by chromosomal instability, a paucity of oncogenic driver mutations and frequent amplifications of upstream activators of signalling pathways.[19,44,45] Within the stomach tumours may also be of the genomically stable or mismatch repair subtype with the final subgroup of EBV positive tumours occurring in the distal stomach.[44,45] Mutational signature analysis of whole genome sequencing data from 129 chemotherapy-naïve OAC samples has revealed three subgroups demonstrating either deficiencies in DNA damage repair, high mutational burden or a C>A/T mutational pattern.[22] The DDR impaired subgroup constitutes 20% of OAC patients and, whilst this is in keeping with a DDIR positive rate of 24%, our analysis has shown no significant overlap

between the two subgroups. Reasons for this discrepancy could include the differing methodologies used to define DNA repair defects between the sequencing and gene expression dataset, the lack of a defined cut-point to call DDR impaired status in the WGS data and the limited sample size. Also, the DDIR assay takes a functional approach, capturing the inflammatory response activated by DNA damage, whereas analysis of the sequencing data assesses the pattern of mutations which occur as a result of loss of DNA repair. Furthermore, Janjigian et al performed prospective sequencing of 295 patients with metastatic oesophago-gastric cancer using a capture-based NGS platform capable of detecting mutations, copy number alterations and selected rearrangements in up to 468 cancer genes. No single mutant allele or gene with a role in DNA repair was associated with immune response and a surrogate marker of homologous recombination (HR) deficiency, termed the large scale transition (LST) score, was not associated with improved progression free survival (HR 0.99, p= 0.947) following first-line platinum-based chemotherapy. Higher LST scores were not observed in patients with response to first-line therapy lasting over 24 months (p= 0.6) and neither did the majority of patients with prolonged responses harbour somatic alterations in know HR genes. Conversely, Smyth et al showed that assessment of Homologous Recombination deficiency using a genomic signature for Loss of Heterozygosity (LOH) derived from an NGS panel could identify a high LOH group of patients with prolonged survival following platinum-based chemotherapy.[46] However, this study was limited by a high attrition rate for LOH inference (47% of samples successfully scored) and small sample numbers. A possible explanation for these results is the limitations imposed by targeted platforms which are unable to detect alterations in genes absent from the panel as well as epigenetic and transcriptional consequences of somatic mutations. Previous studies in breast cancer have shown that while BRCA1/2 mutations may confer sensitivity to DNA-damaging chemotherapy this is not true for all cases as not all mutations may affect DNA repair or may be compensated for by alternate mechanisms.[47] Conversely BRCA1/2 wild-type tumours can possess an

abnormal DNA damage response due to epigenetic silencing of BRCA1/2.[48,49] Therefore, it is likely that the transcriptome based DDIR assay is capable of capturing the downstream effects of genomic and epigenetic changes and so detect a broader range of mechanisms of DDR impairment. It is clear that a subgroup of patients with DNA repair deficiencies exists within OAC and further work is needed to accurately characterise this patient group.

Recently the field of DNA repair biology has enjoyed renewed interest due to its involvement in the immune response to cancer. Increased DNA damage within cancer cells has been shown to generate a highly immunogenic state within the tumours leading to the presence of TILs and the upregulation of suppressors of the immune response, such as PD-L1.[50] Our data indicates a strong association between DDIR positivity and an immunogenic micro-environment. Indeed, our group has demonstrated the role of the cGAS-STING pathway in the response to DNA damage with the resultant upregulation of inflammatory cytokines such as CXCL10 and CCL5 as well as PD-L1.[27] The STING pathway is activated by cytosolic DNA released from the nucleus in response to DNA damage, driving an innate immune type 1 interferon response and a subsequent upregulation of immune checkpoints including PD-L1, a key component of the DDIR signature. Furthermore the cGAS-STING pathway has been shown to be a key player in response to immune checkpoint blockade.[51,52] In keeping with this, we demonstrated increased CD8+ T cell infiltration and PD-L1 expression in DDIR positive oesophageal tumours, both of which have been proposed as predictive biomarkers for immunotherapy agents.[53,54] The presence of a DNA damage deficient subgroup in oesophago-gastric cancer may not only indicate sensitivity to conventional chemotherapy but also response to immune checkpoint targeted agents.

In summary we have developed an array-based classifier using pre-treatment FFPE biopsies to predict benefit from, and response to, neo-adjuvant therapy in resectable OAC. The assay is readily applicable to routine pathological samples with potential for rapid

translation into clinical use. The identification of a subgroup of tumours with deficiencies in their DNA repair mechanisms will enable these patients to be selected for more effective therapy and improve survival outcomes. Also, knowing the underlying biology of these tumours allows the possibility of further enhancing response to therapy through combinations with novel inhibitors of DNA repair and immunotherapy. Overall the DDIR assay enables treatment selection and patient stratification in oesophago-gastric adenocarcinoma and may improve response to therapy, resection rates and survival in this poor prognostic disease.

Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) Consortium:

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ACKNOWLEDGEMENTS

This work was supported by the Gastrointestinal Cancer Research Charitable Fund administered by the Belfast Health and Social Care Trust, the Cancer Research UK Experimental Cancer Medicine Centre Initiative, Invest Northern Ireland and Almac Diagnostics. OCCAMS was funded by a programme grant from Cancer Research UK (RG66287). We thank the Human Research Tissue Bank, which is supported by the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre, from Addenbrooke's Hospital. Additional infrastructure support was provided from the CRUK funded Experimental Cancer Medicine Centre. R.C.F. has programmatic funding from the Medical Research Council and infrastructure support from the NIHR Biomedical Research Centre and the Cambridge Experimental Medicine Centre.

Tissue samples used in this research were received from the Northern Ireland Biobank (NIB) which is funded by HSC Research and Development Division of the Public Health Agency in Northern Ireland and Cancer Research UK through the Belfast Cancer Research UK Centre and the Northern Ireland Experimental Cancer Medicine Centre; additional support was received from the Friends of the Cancer Centre. The Northern Ireland Molecular Pathology Laboratory has received funding from Cancer Research UK, the Friends of the Cancer Centre and the Sean Crummey Foundation.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 721906.

The Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) Study Group is a multicentre UK collaboration.

CONFLICT OF INTEREST

L.A. Knight, A.M. McCavigan, S.M Walker, D.P. Harkin and R.D. Kennedy are employees of Almac Diagnostics and have patent declarations.

G. E. Logan and C. J. Steele are employees of Almac Diagnostics.

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Legends to Figures and Tables

Figure 1: Kaplan-Meier curves stratified by the DDIR assay for (A) relapse-free and (B) overall survival for 273 oesophageal adenocarcinoma patients treated with cisplatin-based neo-adjuvant chemotherapy followed by surgical resection.

Figure 2: Boxplot of DDIR scores grouped by response status

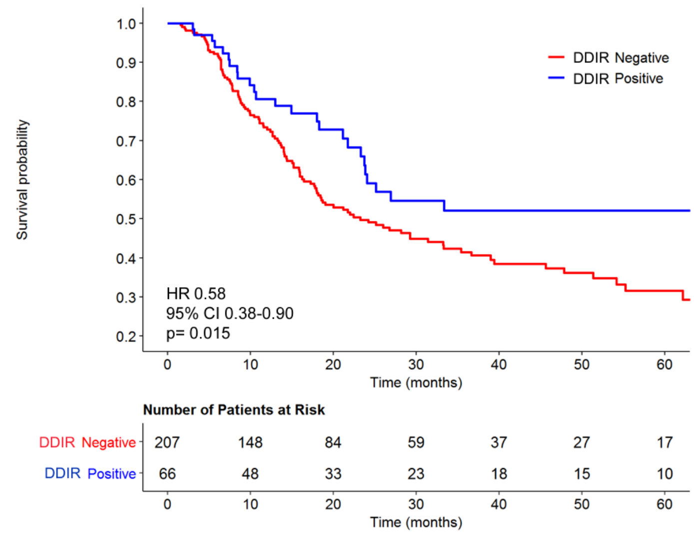
Figure 3: Immunohistochemistry (IHC) images (x10; inset x40) showing absence of CD8+ lymphocytes and PD-L1 staining in DDIR-assay negative tumours. Both intra-tumoural and stromal CD8+ lymphocytes were observed in DDIR assay-positive tumours along with PD-L1 tumours. Scale bar represents 50μM.

Table 1: Association of clinicopathological characteristics with DDIR status in the OAC cohort

Table 2: Multivariable analysis of the predictive value of the DDIR assay adjusted for standard clinicopathological factors available at diagnosis (clinical N stage, clinical T-stage and differentiation).

Table 3. CD8+ intra-tumoural and stromal lymphocytic infiltrate and PD-L1 staining assessed by IHC in DDIR-positive and DDIR-negative tumours.

Figure 1



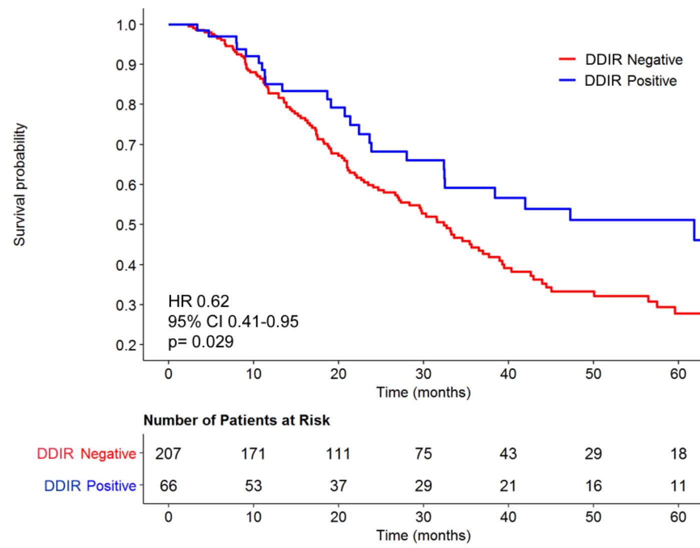


Figure 2

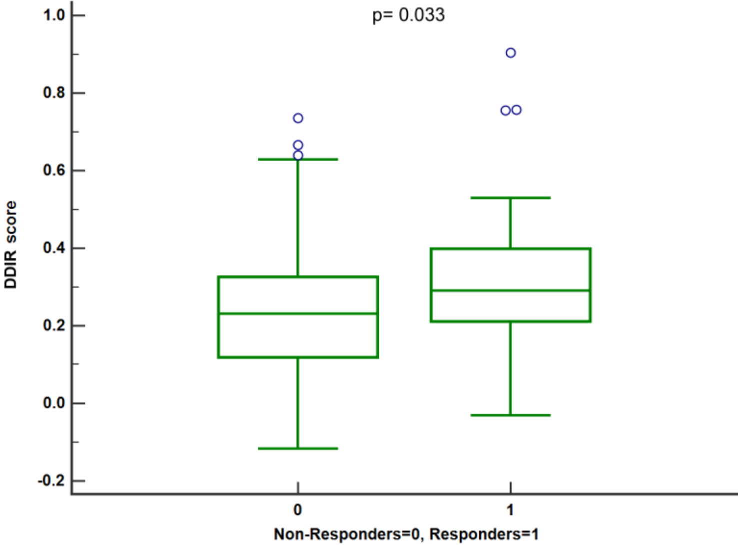


Figure 3

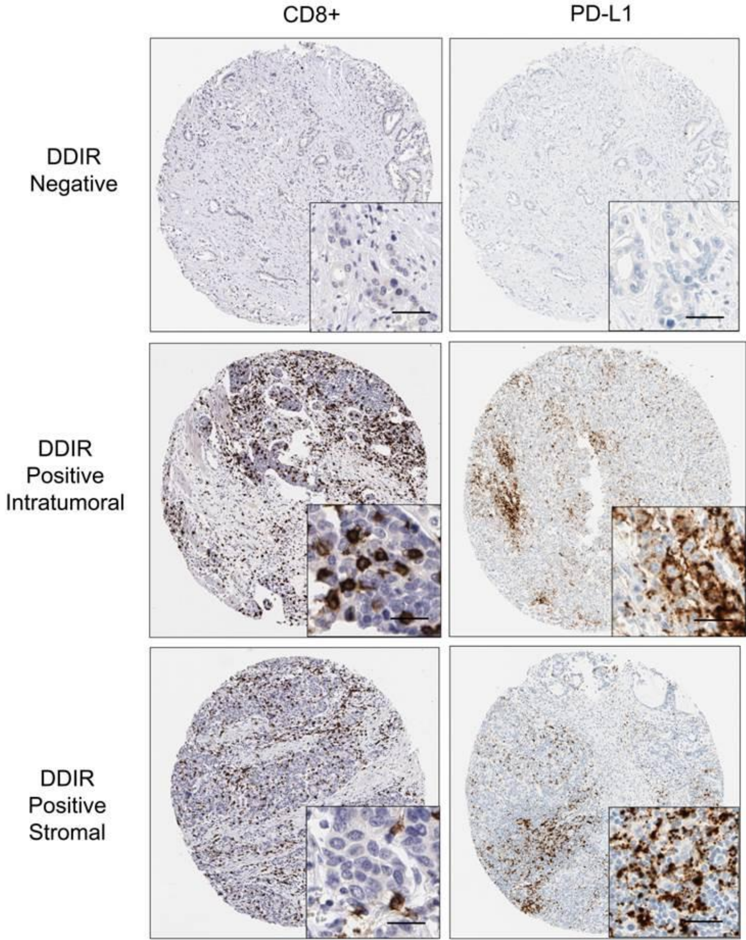


Table 1 Association of clinicopathological characteristics with DDIR status in the OAC cohort

	DDIR Positive (n= 66)		DDIR Negative (n= 207)		p value
	N	%	N	%	
Age					
<60	14	21.2	56	27.1	
60-69	20	30.3	92	44.4	0.035
≥ 70	24	36.4	47	22.7	
Unknown	8	12.1	12	5.8	
Median	66		64		0.049†
Range	41-79		28-83		
Sex					
Male	54	81.8	168	81.2	0.905
Female	12	18.2	39	18.8	
Tumour Site					
Oesophagus	15	22.7	18	8.7	
GOJ, Siewert 1	27	40.9	103	49.8	0.009
GOJ, Siewert 2	14	21.2	64	30.9	
GOJ, Siewert 3	10	15.2	22	10.6	
Clinical T stage					
cT1	1	1.5	3	1.4	
cT2	8	12.1	20	9.7	
cT3	48	72.7	160	77.3	0.936
cT4	2	3	6	2.9	
Unknown	7	10.6	18	8.7	
Clinical N stage					
N0	12	18.2	50	24.2	
N1	39	59.1	121	58.5	
N2	6	9.1	10	4.8	0.378
N3	3	4.5	5	2.4	
Unknown	6	9.1	21	10.1	
Pathological T stage					
ypT0	6	9.1	6	2.9	
ypT1	11	16.7	20	9.7	
ypT2	10	15.2	32	15.5	0.1
ypT3	36	54.5	139	67.1	
ypT4	3	4.5	10	4.8	
Pathological N stage					
ypN0	33	50	69	33.3	
ypN1	9	13.6	52	25.1	0.026
ypN2	16	24.2	42	20.3	
ypN3	8	12.1	44	21.3	
Lymph Node Yield					
≥ 15	45	68.2	151	72.9	
< 15	21	31.8	55	26.6	0.433
Unknown	0	0	1	0.5	
Median	21.5		21		0.863†
Range	6-41		6-62		
Differentiation					
Well	4	6.1	3	1.4	
Moderate	16	24.2	74	35.7	0.044
Poor	40	60.1	121	58.5	
Unknown	6	9.1	9	4.3	
Lymphovascular Invasion					
Negative	25	37.9	61	29.5	
Positive	39	59.1	139	67.1	0.222
Unknown	2	3	7	3.4	
Circumferential Resection Margin					
Negative	47	71.2	111	53.6	
Positive	15	22.7	85	41.1	0.007
Unknown	4	6.1	11	5.3	
Neo-Adjuvant chemotherapy					
CFU/CX	12	18.2	33	15.9	
ECF/X	52	78.8	168	81.2	0.89
Oxaliplatin/X	1	1.5	4	1.9	
Unknown	1	1.5	2	1	
Adjuvant Chemotherapy Received					
No	12	18.2	48	23.2	0.448
Yes	26	39.4	75	36.2	

Unknown	28	42.4	84	40.6	
Pathological Response					
Responder	11	16.7	14	6.8	
Non-Responder	45	68.2	158	76.3	0.025
Unknown	10	15.2	35	16.9	

†Mann-Whitney *U* Test

Table 2 Multivariate analysis and combined model of clinicopathological factors, DDIR status, relapse-free and overall survival in OAC.

	Relapse-free Survival			Overall Survival		
	HR	95% CI	p value	HR	95% CI	p value
Multivariate Model						
DDIR Positive	0.61	0.38-0.98	0.042	0.52	0.31-0.88	0.015
Clinical T stage (T1/2 v 3/4)	1.08	0.56-2.09	0.810	1.05	0.55-2.03	0.876
Clinical N stage (N0 v 1/2/3)	1.67	1.04-2.67	0.033	1.51	1.94-2.42	0.088
Differentiation (Well/Moderate v Poor)	1.32	0.91-1.92	0.146	1.43	0.97-2.10	0.071

Table 3. CD8+ intra-tumoural and stromal lymphocytic infiltrate and PD-L1 staining assessed by IHC in DDIR-positive and DDIR-negative tumours.

	DDIR Positive (n =24)		DDIR Negative (n= 102)		p value
	N	%	N	%	
Intra-tumoural					
PDL1					
≥1%	7	29.2	10	9.8	0.02
<1%	17	70.8	92	90.2	
≥5%	3	12.5	2	2	0.047
<5%	21	70.8	100	90.2	
CD8+					
3	1	4.2	0	0	<0.001
2	4	16.7	1	1	
1	14	13.7	63	61.7	
0	5	4.9	38	37.2	
Stromal					
PDL1					
≥1%	17	70.8	52	51	0.11
<1%	7	29.2	50	49	
≥5%	8	33.3	12	11.8	0.025
<5%	16	66.7	90	88.2	
CD8+					
3	8	33.3	10	9.8	0.026
2	8	33.3	45	44.1	
1	8	33.3	44	43.1	
0	0	0	3	2.9	

Supplementary Tables

Supplementary Table 1 Comparison of the reporting of the DDIR assay as a predictive marker in oesophageal adenocarcinoma with the REMARK guidelines

REMARK Guidelines Criteria	DDIR in OAC
INTRODUCTION	
State the marker examined, study objectives and pre-specified hypothesis	The marker examined was the DNA Damage Response Deficiency assay. We assessed the ability of a clinically validated DNA Damage Response Deficiency (DDIR) assay to predict prognosis following DNA damaging neo-adjuvant chemotherapy in oesophageal adenocarcinoma.
MATERIALS AND METHODS	
Patients	
Describe the characteristics (for example, disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria. Describe the treatments received and how chosen	n=273 OAC patients treated with neo-adjuvant chemotherapy and surgical resection, n= 70 oesophageal adenocarcinoma patients treated by surgery alone (see Methods section)
Specimen Characteristics	
Type of biological material used, methods of preservation and storage	Formalin fixed paraffin embedded (FFPE) endoscopic biopsies and resection specimens. Fresh frozen chemotherapy-naïve resection specimens.
Assay Methods	
Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	DNA Damage Response Deficiency Assay (see Methods section and Mulligan et al J Natl Cancer Inst. 2014 Jan;106(1))
Study Design	
State the method of case selection, including whether prospective or retrospective and whether stratification or matching (for example, by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	See Methods section
Precisely define all clinical endpoints	See Statistical Analysis section
List all candidate variables initially examined or considered for inclusion in models	clinical T stage, clinical N Stage, tumour grade, DDIR status
Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	Assuming a marker positive rate of 21% (estimated from preliminary data) a sample set of 273 patients has 80% power to detect a Hazard Ratio (HR) of 0.5/2.
Statistical Analysis Methods	
Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	See Methods section
Clarify how marker values were handled and describe methods used for cutpoint determination	See Methods section
RESULTS	
Data	
Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the number of patients and the number of events.	Clinicopathological characteristics of the oesophageal adenocarcinoma datasets are described and the flow of patients outlined in Supplementary Figure 1
Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values.	See Supplementary Tables 2, 3, 4 and 5.
Analysis and presentation	
Show the relation of the marker to standard prognostic variables	See Table 1, Supplementary Tables 8 and 9.
Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (for example, hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analysed. For the effect of a tumour marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	See Supplementary Table 6.
For key multivariable analyses, report estimated effects (for example, hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	See Table 2.
Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	See Supplementary Table 6.
If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	See Results section
DISCUSSION	
Interpret the results in the context of the pre-specified hypothesis, other relevant studies and limitations Discuss implications for future research and clinical value	See Discussion

Supplementary Table 2 Clinicopathological characteristics of the OAC cohort

	OAC (n= 273)	
	N	**%**
Age		
<60	70	25.6
60-69	112	41
≥ 70	71	26
Unknown	20	7.3
Median	64	
Range	28-83	
Sex		
Male	222	81.3
Female	51	18.7
Tumour Site		
Oesophagus	33	12.1
GOJ, Siewert 1	130	47.6
GOJ, Siewert 2	78	28.6
GOJ, Siewert 3	32	11.7
Clinical T stage		
cT1	4	14.7
cT2	28	10.3
cT3	208	76.2
cT4	8	29.3
Unknown	25	9.2
Clinical N stage		
N0	62	22.7
N1	160	58.6
N2	16	5.9
N3	8	2.9
Unknown	27	9.9
Pathological T stage		
ypT0	12	44
ypT1	31	11.4
ypT2	42	15.4
ypT3	175	64.1
ypT4	13	4.8
Pathological N stage		
ypN0	102	37.4
ypN1	61	22.3
ypN2	58	21.2
ypN3	52	19
Differentiation		
Well	7	2.6
Moderate	90	33
Poor	161	59
Unknown	15	5.5
Lymphovascular Invasion		
Negative	86	31.5
Positive	178	65.2
Unknown	9	3.3
Circumferential Resection Margin		
Negative	158	57.9
Positive	100	36.6
Unknown	15	5.5
Neo-Adjuvant chemotherapy		
CFU/CX	45	16.5
ECF/X	220	80.6
Oxaliplatin/X	5	1.8
Unknown	3	1.1

Supplementary Table 3 Association of clinicopathological characteristics of the surgery alone OAC cohort with DDIR status

	OAC (n= 57)		DDIR Positive (n= 31)		DDIR Negative (n= 26)		p value
	N	%	N	%	N	%	
Sex							
Male	40	70.2	21	67.7	19	73.1	0.775
Female	17	29.8	10	32.3	7	26.9	
Pathological T stage							
pT0	1	1.8	0	0	1	3.8	
pT1	4	7	3	9.7	1	3.8	
pT2	16	28.1	6	19.4	10	38.5	0.287
pT3	35	61.4	21	67.7	14	53.8	
pT4	1	1.8	1	3.2	0	0	
Pathological N stage							
pN0	14	24.6	7	22.6	7	26.9	
pN1	35	61.4	19	61.3	16	61.5	0.809
pN2	7	12.3	4	12.9	3	11.5	
pN3	1	1.8	1	3.2	0	0	
Pathological M stage							
pM0	55	96.5	30	96.8	25	96.2	0.899
pM1	2	3.5	1	3.2	1	3.8	
Differentiation							
Well	4	7	2	6.5	2	7.7	
Moderate	27	47.4	15	48.4	12	46.2	0.976
Poor	26	45.6	14	45.2	12	46.2	
Circumferential Resection Margin							
Negative	21	36.8	9	29	12	46.2	0.182
Positive	36	63.2	22	71	14	53.8	

Supplementary Table 4 Comparison of the Clinicopathological characteristics of the OAC cohort and Sub-cohorts

	OAC (n= 273)		OAC TMA (n= 126)		OAC WGS (n= 44)		p value
	N	%	N	%	N	%	
Age							
<60	70	25.6	36	28.6	12	27.3	
60-69	112	41	60	47.6	12	27.3	0.662
≥ 70	71	26	30	23.8	11	25	
Unknown	20	73.3			9	20.5	
Median	64		63		65		0.473
Range	28-83		28-83		41-79		
Sex							
Male	222	81.3	97	77	40	90.9	0.126
Female	51	18.7	29	23	4	9.1	
Tumour Site							
Oesophagus	33	12.1	19	15.1	4	9.1	
GOJ, Siewert 1	130	47.6	66	52.4	20	45.5	0.609
GOJ, Siewert 2	78	28.6	31	24.6	16	36.4	
GOJ, Siewert 3	32	11.7	10	7.9	4	9.1	
Clinical T stage							
cT1	4	14.7	2	1.6	1	2.3	
cT2	28	10.3	8	6.3	6	13.6	0.738
cT3	208	76.2	103	81.7	32	72.7	
cT4	8	29.3	2	1.6	1	2.3	
Unknown	25	9.2	11	8.7	4	9.1	
Clinical N stage							
N0	62	22.7	30	23.8	8	18.2	
N1	160	58.6	73	57.9	25	56.8	0.01
N2	16	5.9	2	1.6	8	18.2	
N3	8	2.9	2	1.6	3	6.8	
Unknown	27	9.9	19	15.1	0	0	
Pathological T stage							
ypT0	12	44	3	2.4	2	4.5	
ypT1	31	11.4	10	7.9	7	15.9	0.356
ypT2	42	15.4	22	17.5	2	4.5	
ypT3	175	64.1	86	68.3	29	65.9	
ypT4	13	4.8	5	4	4	9.1	
Pathological N stage							
ypN0	102	37.4	42	33.3	13	29.5	
ypN1	61	22.3	27	21.4	10	22.7	0.847
ypN2	58	21.2	31	24.6	9	20.5	
ypN3	52	19	26	20.6	12	27.3	
Differentiation							
Well	7	2.6	2	1.6	0	0	
Moderate	90	33	49	38.9	15	34.1	0.726
Poor	161	59	74	58.7	26	59.1	
Unknown	15	5.5	1	0.8	3	6.8	
Lymphovascular Invasion							
Negative	86	31.5	41	32.5	12	27.3	
Positive	178	65.2	84	66.7	30	68.2	0.865
Unknown	9	3.3	1	0.8	2	4.5	
Circumferential Resection Margin							
Negative	158	57.9	67	53.2	21	47.7	
Positive	100	36.6	58	46	12	27.3	0.311
Unknown	15	5.5	1	0.8	11	25	
Neo-Adjuvant chemotherapy							
CFU/CX	45	16.5	1	0.8	15	34.1	
ECF/X	220	80.6	123	97.6	24	54.5	<0.0001
Oxaliplatin/X	5	1.8	2	1.6	3	6.8	
Unknown	3	1.1	0	0	2	4.5	

†Kruskall Wallis test

TMA- Tissue Microarray, WGS- Whole Genome Sequencing

Supplementary Table 5 Univariate analysis of clinicopathological factors, DDIR status, relapse-free and overall survival in OAC.

	Relapse-free Survival			Overall Survival		
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
DDIR status (Pos vs Neg)	0.58	0.38-0.90	0.015	0.62	0.41-0.95	0.029
Age	0.99	0.97-1.01	0.193	1.00	0.98-1.02	0.950
Gender	0.76	0.49-1.18	0.222	0.68	0.44-1.07	0.092
Clinical T stage (T1/2 v T3/4)	1.80	0.99-3.27	0.054	1.66	0.93-2.96	0.084
Clinical N stage (N0 v N1/2/3)	1.68	1.09-2.59	0.019	1.59	1.03-2.45	0.038
Lymph Node Yield (<15 vs ≥15)	0.94	0.66-1.39	0.847	1.02	0.7-1.48	0.916
Pathological T stage (T0/1/2 v T3/4)	3.46	2.22-5.39	<0.001	3.19	2.08-4.90	<0.001
Pathological N stage (N0 vs N1/2/3)	4.05	2.68-6.14	<0.001	4.07	2.68-6.19	<0.001
Differentiation (Well/Moderate vs Poor)	1.41	1.01-1.97	0.045	1.56	1.12-2.19	0.010
Lymphovascular invasion (Neg vs Pos)	2.56	1.70-3.86	<0.001	2.88	1.89-4.41	<0.001
Circumferential Resection Margin (Neg vs Pos)	3.22	2.27-4.58	<0.001	3.26	2.30-4.63	<0.001

Supplementary Table 6 Correlation of DDIR status and Mutational Signature Subgroups for 44 OAC cases with matched gene expression and WGS data

	DDIR Positive n= 13	**DDIR Negative** n= 31	**Chi-squared**
C>A Dominant	2	6	
DDRi	3	9	0.83
Mutagenic	8	16	

Supplementary Table 7 Genes upregulated in DDIR positive relative to DDIR negative patients.

Gene	Description	Fold-Change	p-value
IDO1	indoleamine 2,3-dioxygenase 1	7.04	2.21E-31
CXCL9	chemokine (C-X-C motif) ligand 9	5.50	1.19E-20
CXCL13	chemokine (C-X-C motif) ligand 13	4.58	2.77E-26
GBP5	guanylate binding protein 5	4.51	1.37E-25
ART3	ADP-ribosyltransferase 3	3.73	8.74E-26
CXCL10	chemokine (C-X-C motif) ligand 10	3.65	1.19E-29
CPNE4	copine IV	3.37	4.44E-15
GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1	3.32	2.23E-20
CXCL11	chemokine (C-X-C motif) ligand 11	3.24	2.17E-23
IFI44L	interferon-induced protein 44-like	2.92	4.90E-11
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	2.67	6.15E-06
IGLV2-23	immunoglobulin lambda variable 2-23	2.56	4.98E-07
GBP4	guanylate binding protein 4	2.54	1.24E-29
RSAD2	radical S-adenosyl methionine domain containing 2	2.45	9.55E-10
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	2.43	4.69E-12
GBP1	guanylate binding protein 1, interferon-inducible	2.39	6.19E-16
TRAC	T cell receptor alpha constant	2.35	7.49E-11
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	2.31	1.85E-18
C1orf186	chromosome 1 open reading frame 186	2.30	3.18E-06
CCL5	chemokine (C-C motif) ligand 5	2.29	1.29E-14
STAT1	signal transducer and activator of transcription 1	2.27	2.11E-24
AIM2	absent in melanoma 2	2.25	6.46E-12
OAS2	2'-5'-oligoadenylate synthetase 2	2.24	2.80E-08
CCL8	chemokine (C-C motif) ligand 8	2.23	2.79E-07
MS4A1	membrane-spanning 4-domains, subfamily A, member 1	2.23	1.56E-08
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	2.22	8.63E-14
CD38	CD38 molecule	2.19	2.34E-12
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	2.17	3.43E-12
BIRC3	baculoviral IAP repeat containing 3	2.16	2.67E-08
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.15	1.16E-18
EPSTI1	epithelial stromal interaction 1 (breast)	2.14	7.37E-11
IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	2.13	9.97E-06
CFB	complement factor B	2.13	9.95E-07
BATF2	basic leucine zipper transcription factor, ATF-like 2	2.12	7.61E-22
IFIH1	interferon induced with helicase C domain 1	2.11	9.54E-09
CD8A	CD8a molecule	2.11	1.01E-18
SAMD9L	sterile alpha motif domain containing 9-like	2.11	1.23E-09
WARS	tryptophanyl-tRNA synthetase	2.10	2.22E-19
HLA-F	major histocompatibility complex, class I, F	2.10	3.80E-06
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	2.07	0.000114567
XAF1	XIAP associated factor 1	2.05	2.17E-10
CD274/PD-L1	CD274 molecule/Programmed Death Ligand 1	2.03	1.20E-12
UBE2L6	ubiquitin-conjugating enzyme E2L 6	2.03	1.81E-19
FAM26F	family with sequence similarity 26, member F	2.02	1.72E-12
IFITM2	interferon induced transmembrane protein 2	2.01	3.83E-05

Supplementary Table 8 Biological Processes enriched in the DDIR positive relative to DDIR negative patients

Gene Ontology Term	p value	Fold Enrichment	FDR
GO:0006958 Complement activation, classical pathway	0.00015	3.815	0.256
GO:0006911 Phagocytosis, engulfment	0.00016	6.640	0.277
GO:0006956 Complement activation	0.00019	4.007	0.329
GO:0050871 Positive regulation of B cell activation	0.00021	7.822	0.359
GO:0006910 Phagocytosis, recognition	0.00032	7.263	0.553
GO:0050853 B cell receptor signaling pathway	0.00048	4.842	0.833
GO:0042742 Defense response to bacterium	0.00432	2.605	7.301
GO:0038096 Fc-gamma receptor signaling pathway involved in phagocytosis	0.00438	2.745	7.394
GO:2000105 Positive regulation of DNA-dependent DNA replication	0.00346	29.052	5.882
GO:0003323 Type B pancreatic cell development	0.00542	10.564	9.075
GO:0040007 Growth	0.01467	5.188	22.807
GO:0019083 Viral transcription	0.01506	2.594	23.335
GO:0019083 Viral transcription	0.01506	2.594	23.335
GO:0006606 Protein import into nucleus	0.04857	3.005	58.188
GO:0006405 RNA export from nucleus	0.04004	3.169	51.111
GO:0035455 Response to interferon-alpha	0.04418	8.716	54.673

Supplementary Table 9 Correlation of clinicopathological characteristics with PD-L1 status in the OAC cohort

	Intra-tumoural					Stromal				
	PD-L1 ≥ 5% (n= 5)		PD-L1 < 5% (n= 121)		p value	PD-L1 ≥ 5% (n= 20)		PD-L1 < 5% (n= 106)		p value
	N	%	N	%		N	%	N	%	
Age										
<60	1	20	35	28.9		5	25	31	29.2	
60-69	2	40	58	47.9	0.68	9	45	51	48.1	0.77
≥ 70	2	40	28	23.1		6	30	24	22.6	
Median	68		63		0.24†	64		63		0.802†
Range	59-78		28-83			28-78		44-83		
Sex										
Male	5	100	92	76	0.212	18	90	79	74.5	0.132
Female	0	0	29	24		2	10	27	25.5	
Tumour Site										
Oesophagus	0	0	19	15.7		4	20	15	14.2	
GOJ, Siewert 1	4	80	62	51.2	0.572	12	60	54	50.9	0.616
GOJ, Siewert 2	1	20	30	24.8		3	15	28	26.4	
GOJ, Siewert 3	0	0	10	8.3		1	5	9	8.5	
Clinical T stage										
cT1	1	20	1	0.8		1	5	1	0.9	
cT2	0	0	8	6.6		2	10	6	5.7	
cT3	3	60	100	82.6	<0.001	14	70	89	84	0.218
cT4	1	20	1	0.8		1	5	1	0.9	
Unknown	0	0	11	9.1		2	10	9	8.5	
Clinical N stage										
N0	0	0	30	24.8		4	20	26	24.5	
N1	4	80	69	57		13	65	60	56.6	
N2	0	0	2	1.7	0.586	0	0	2	1.9	0.776
N3	0	0	2	1.7		0	0	2	1.9	
Unknown	1	20	18	14.9		3	15	16	15.1	
Neo-Adjuvant chemotherapy										
CFU/CX	0	0	1	0.8	0.838	0	0	1	0.9	0.663
ECF/X	5	100	120	99.2		20	100	105	99.1	
PET Response										
Responder	3	60	38	31.4		7	35	32	30.2	
Non-Responder	1	20	54	44.6	0.386	11	55	46	43.4	0.282
Unknown	1	20	29	24		2	10	28	26.4	
Pathological Response										
Responder	0	0	9	7.4	0.76	3	15	6	5.7	0.149
Non-Responder	5	100	109	90.1		17	85	97	91.5	

Unknown	0	0	3	2.5		0	0	3	2.8	
Pathological T stage										
ypT0	0	0	3	2.5		1	5	2	1.9	
ypT1	1	20	9	7.4		4	20	6	5.7	
ypT2	1	20	21	17.4	0.852	6	30	16	15.1	0.04
ypT3	3	60	83	68.6		9	45	77	72.6	
ypT4	0	0	5	4.1		0	0	5	4.7	
Pathological N stage										
ypN0	3	60	39	32.2		10	50	32	30.2	
ypN1	1	20	26	21.5	0.525	5	25	22	20.8	0.229
ypN2	1	20	30	24.8		3	15	28	26.4	
ypN3	0	0	26	21.5		2	10	24	22.6	
Differentiation										
Well	0	0	2	1.7		1	5	1	0.9	
Moderate	0	0	49	40.5	0.166	5	25	44	41.5	0.207
Poor	5	100	69	57		13	65	61	57.5	
Unknown	0	0	1	0.8		1	5	0	0	
Lymphovascular Invasion										
Negative	2	40	80	66.1		10	50	31	29.2	
Positive	3	60	40	33.1	0.219	10	50	74	69.8	0.074
Unknown	0	0	1	0.8		0	0	1	0.9	
Circumferential Resection Margin										
Negative	2	40	65	53.7		13	65	54	50.9	
Positive	3	60	55	45.5	0.534	7	35	51	48.1	0.265
Unknown	0	0	1	0.8		0	0	1	0.9	

†Mann-Whitney *U* Test

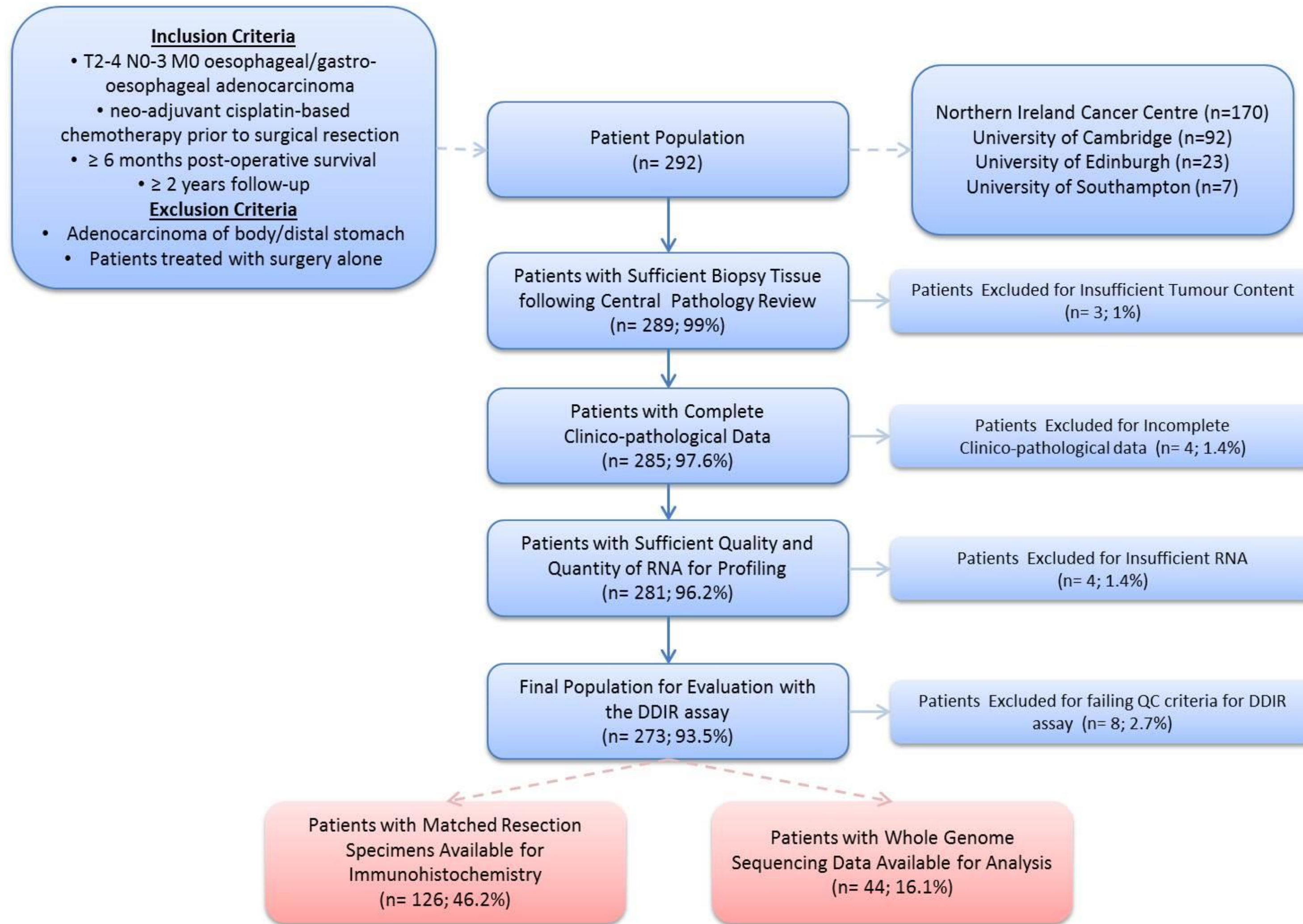
Supplementary Table 10 Correlation of clinicopathological characteristics with CD8 staining in the OAC cohort

	Intra-tumoural					Stromal				
	0	**1**	**2**	**3**	**p value**	**0**	**1**	**2**	**3**	**p value**
	n= 43	**n= 77**	**n= 5**	**n= 1**		**n= 3**	**n= 52**	**n= 53**	**n= 18**	
Age										
<60	11	24	0	1		0	18	15	3	
60-69	23	35	2	0	0.269	2	22	28	8	0.46
≥ 70	9	18	3	0		1	12	10	7	
Median	63	63	71	47	0.225[†]	64	63	63	67	0.451[†]
Range	48-78	28-83	61-78	N/A		62-72	28-83	44-78	47-78	
Sex										
Male	33	58	5	1	0.59	1	43	38	15	0.143
Female	10	19	0	0		2	9	15	3	
Tumour Site										
Oesophagus	5	13	1	0		0	8	8	3	
GOJ, Siewert 1	28	33	4	1	0.421	2	30	24	10	0.463
GOJ, Siewert 2	8	23	0	0		0	13	15	3	
GOJ, Siewert 3	2	8	0	0		1	1	6	2	
Clinical T stage										
cT1	0	1	1	0		0	1	0	1	
cT2	2	6	0	0		0	3	4	1	
cT3	37	61	4	1	0.284	1	42	46	14	0.124
cT4	0	2	0	0		0	1	1	0	
Unknown	4	7	0	0		2	5	2	2	
Clinical N stage										
N0	8	22	0	0		1	8	0	3	
N1	28	40	4	1		0	32	18	12	
N2	1	1	0	0	0.912	0	1	29	0	<0.001
N3	0	2	0	0		0	2	1	0	
Unknown	6	12	1	0		2	9	5	3	
Neo-Adjuvant chemotherapy										
CFU/CX	0	1	0	0	0.887	0	0	0	1	0.109
ECF/X	43	76	5	1		3	52	53	17	
PET Response										
Responder	20	32	4	1		2	27	18	10	
Non-Responder	16	22	1	0	0.314	1	19	17	2	0.051
Unknown	7	23	0	0		0	6	18	6	
Pathological Response										
Responder	4	5	0	0	0.881	1	3	4	1	0.644
Non-Responder	37	71	5	1		2	48	48	16	

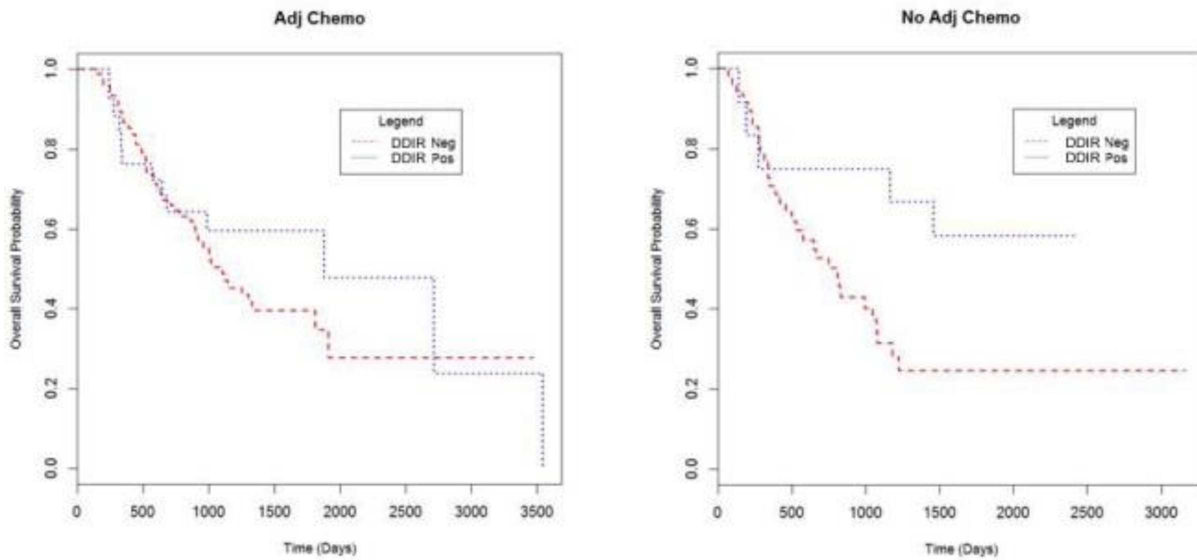
Unknown	2	1	0	0		0	1	1	1	
Pathological T stage										
ypT0	2	1	0	0		0	0	1	1	
ypT1	3	7	0	0		1	5	4	1	
ypT2	6	13	3	0	0.341	0	8	10	4	0.835
ypT3	28	55	2	1		2	36	36	12	
ypT4	4	1	0	0		0	3	2	0	
Pathological N stage										
ypN0	10	29	3	0		1	15	19	7	
ypN1	11	14	1	1	0.211	1	9	12	5	0.674
ypN2	9	22	0	0		0	13	13	5	
ypN3	13	12	1	0		1	15	9	1	
Differentiation										
Well	0	2	0	0		0	0	2	0	
Moderate	19	30	0	0	0.701	2	16	26	5	0.093
Poor	24	44	5	1		1	36	25	12	
Unknown	0	1	0	0		0	0	0	1	
Lymphovascular Invasion										
Negative	9	30	2	0		2	14	18	7	
Positive	34	46	3	1	0.475	1	38	34	11	0.654
Unknown	0	1	0	0		0	0	1	0	
Circumferential Resection Margin										
Negative	19	44	3	1		2	20	32	13	
Positive	24	32	2	0	0.717	1	31	21	5	0.155
Unknown	0	1	0	0		0	1	0	0	

†..Kruskall Wallis Test

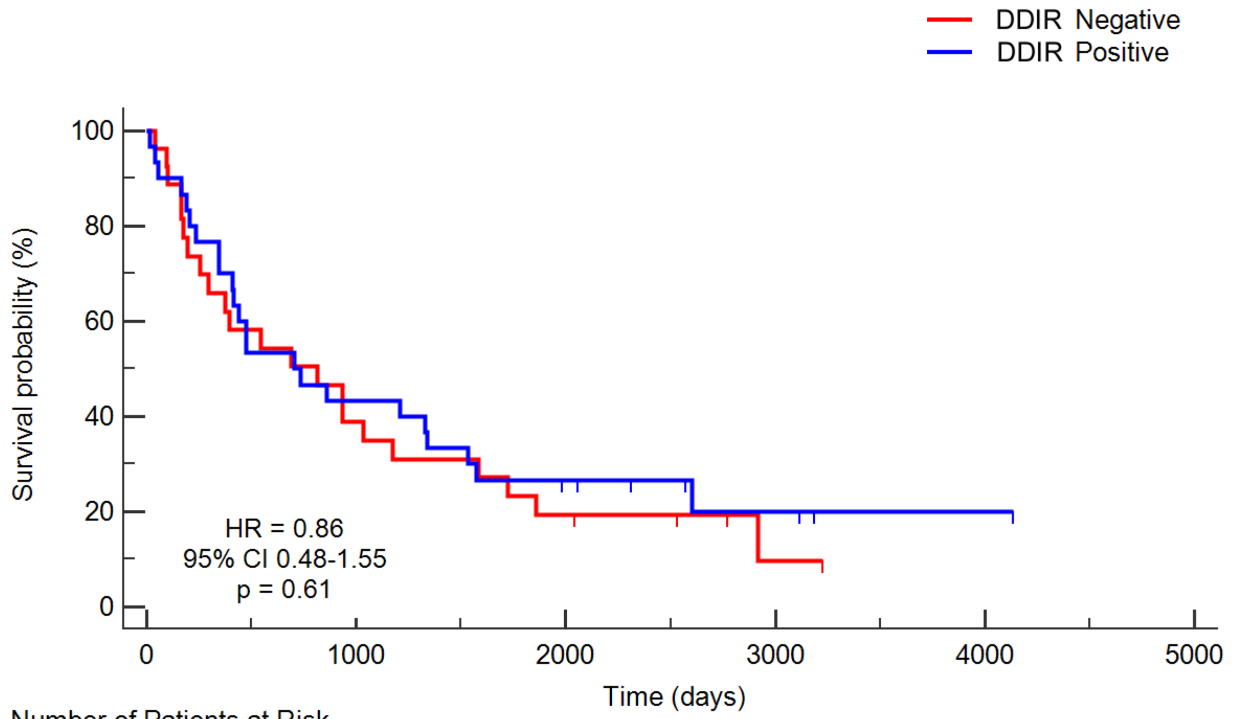
Supplementary Figures



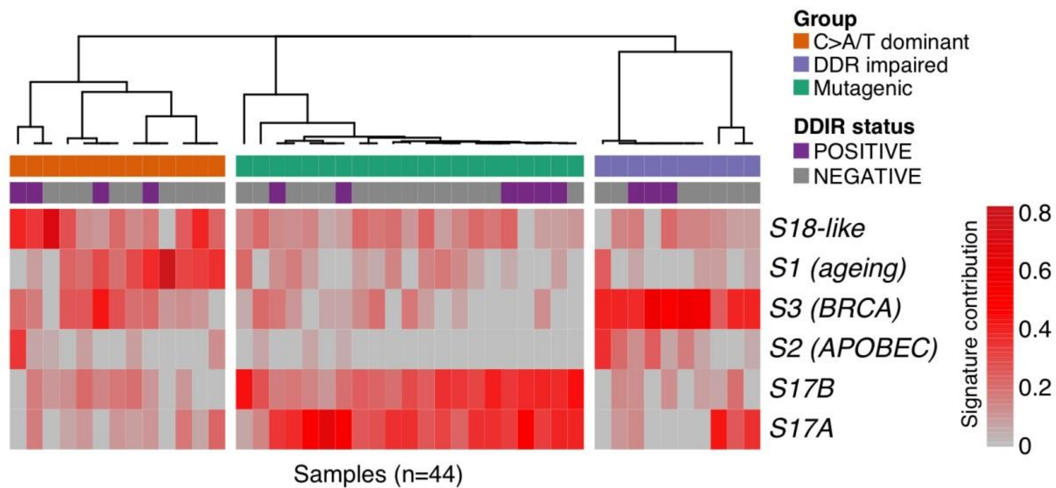
Supplementary Figure 1- REMARK diagram



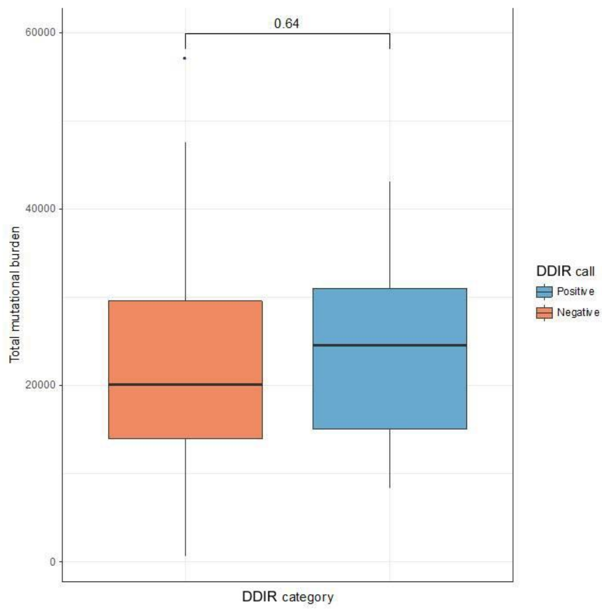
Supplementary Figure 2: Kaplan-Meier curves stratified by the DNA Damage Immune Response (DDIR) assay for overall survival for 101 OAC patients treated with adjuvant chemotherapy following surgical resection and 60 OAC patients who did not receive further chemotherapy following surgical resection.

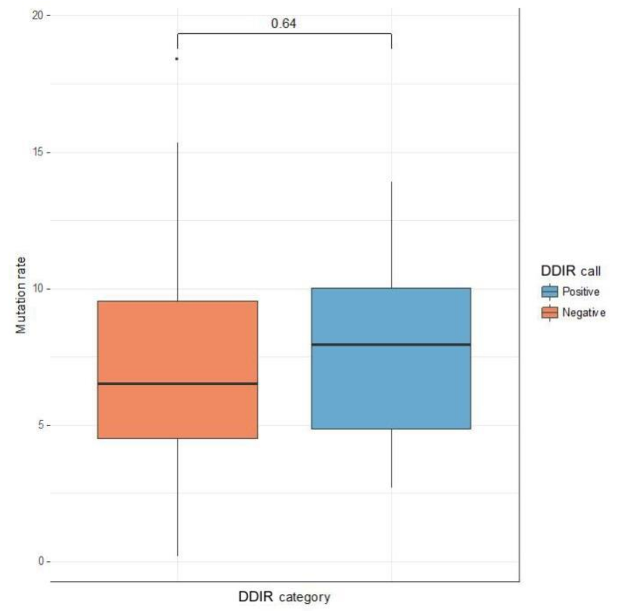


Supplementary Figure 3: Kaplan-Meier curves stratified by the DNA Damage Immune Response (DDIR) assay for overall survival for 57 oesophageal adenocarcinoma patients treated with surgical resection alone.



Supplementary Figure 4: Mutational signature-based clustering of 44 OAC patients analysed by whole genome sequencing. The strength of the exposure to each mutational process associated with distinct risk factors of cancer (0-100%) was calculated using the non-negative matrix factorization (NMF) methodology and hierarchical clustering was used to group the samples based on their mutational signature profiles. Samples were assigned to one of three subgroups reported in Secrier, Li et al, Nat Genet 2016 based on the dominant mutational process in the respective genome as follows: C>A/T dominant (S18-like/S1-ageing; 30%), DDR Impaired (S3-BRCA; 23%) and Mutagenic (S17A/B; 47%). For cases where more than one tumour sample had been whole-genome sequenced, the sample with the highest tumour purity (estimated by ASCAT) was used in the analysis. DDIR status is annotated for each sample.





Supplementary Figure 5: Boxplots of (A) Tumour Mutational Burden and (B) Mutational Rate grouped by DDIR status