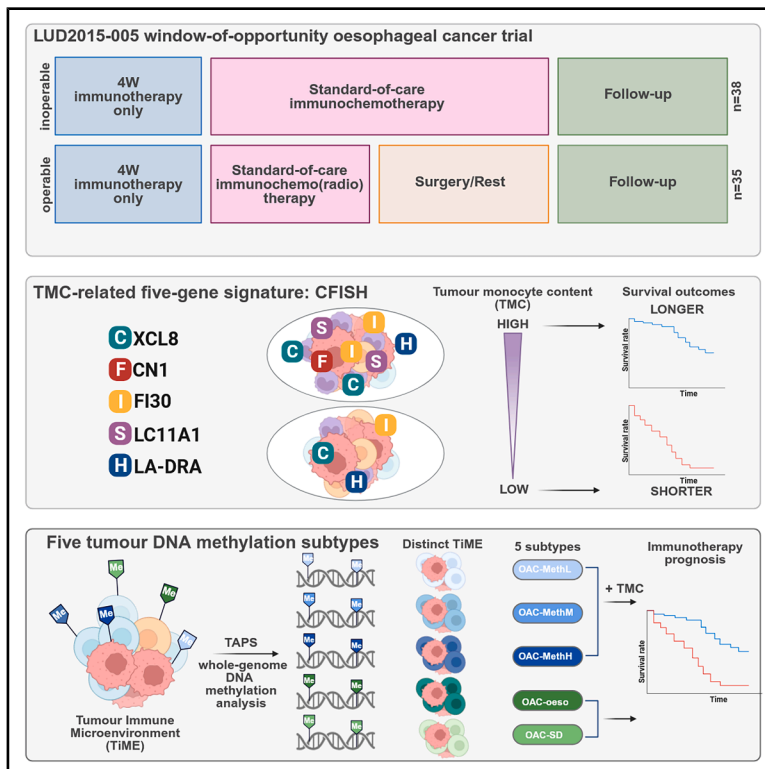


Tumor DNA methylation subtypes and expression levels of CFISH predict response and overall survival to immunochemotherapy

Graphical abstract



Highlights

- Neoadjuvant ICI-based therapy of operable esophageal cancer improved outcomes
- A tumor monocyte-related five-gene signature CFISH predicts esophageal cancer survival
- Tumor DNA methylation classifies esophageal adenocarcinoma into five distinct subtypes
- Tumor DNA methylation subtypes stratify tumor immune microenvironment and ICI response

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In brief

Clinically implementable biomarkers predicting immunochemotherapy response are lacking. Fuchs et al. comprehensively molecularly profiled longitudinal and multi-sites tissue/tumor samples from 73 esophageal cancer patients in LUD2015-005 trial receiving 4-week immunotherapy priming prior to combination-therapy and identified TMC-related five-gene signature CFISH and DNA methylation subtypes that predict patient overall survival to immunochemotherapy.



Translation to Patients

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Article

Tumor DNA methylation subtypes and expression levels of CFISH predict response and overall survival to immunochemotherapy

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CONTEXT AND SIGNIFICANCE Identifying responding patients is a key challenge in cancer therapy. Immunotherapy targets immune cells that surround cancer cells. Esophageal cancer (OC) has poor treatment outcomes, which have improved by adding immunotherapy to chemotherapy. LUD2015-005 phase 1/2 is one of the first trials treating OC patients with immune priming, followed by immunochemotherapy, allowing us to correlate immune responses with long-term clinical outcomes. Detailed analysis of tumors before and after starting immunotherapy shows how the immune cells change and how this might predict who benefits from treatment. Specifically, we identified a five-gene-signature (CFISH) and DNA methylation subtypes as predictive biomarkers for overall survival, providing a much-needed readout of the tumor immune microenvironment (TiME) to stratify and predict patient response to immunochemotherapy.

SUMMARY

Background: Predicting patient response to immunochemotherapy is vital but challenging and poorly studied in the (neo)adjuvant setting. We showed that tumor monocyte content (TMC) predicts overall survival of advanced esophageal cancer (OC) in response to immunochemotherapy. We report clinical outcomes of 35 operable OC patients receiving ICI-based therapy and molecular profiling of the unique phase-2 LUD2015-005 trial treating 73 OC patients (38 advanced/inoperable and 35 operable) in the (neo)adjuvant setting.

Methods: LUD2015-005 trial patients were treated with 4-week immune checkpoint inhibitor (ICI)-only before combination with standard of care. Paired and longitudinally collected biopsies of tumor and multiple adja-

cent normal sites were bi-dissected to extract DNA and RNA. Whole-genome, methylome, single-cell and bulk RNA sequencing data generated from the entire LUD2015-005 cohort were used for cell-deconvolution and data integration analysis.

Findings: Neoadjuvant ICI-based combination therapy of operable OC in the LUD2015-005 trial revealed improved overall outcomes. Tumor monocytes contribute to tumor immune microenvironment (TiME) remodeling and associate with tumor reactive CD8⁺ T cells upon ICI+ chemotherapy/chemoradiotherapy. We identified five DNA-methylation subtypes with distinct TiME and a TMC-related five-gene signature CFISH (CXCL8, FCN1, IFI30, SLC11A1, and HLA-DRA) as highly predictive biomarkers of overall survival in OC and beyond.

Conclusions: These findings highlight the importance of the myeloid compartment in ICI response and the potential of CFISH and tumor DNA methylation in patient stratification for immunochemotherapy.

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INTRODUCTION

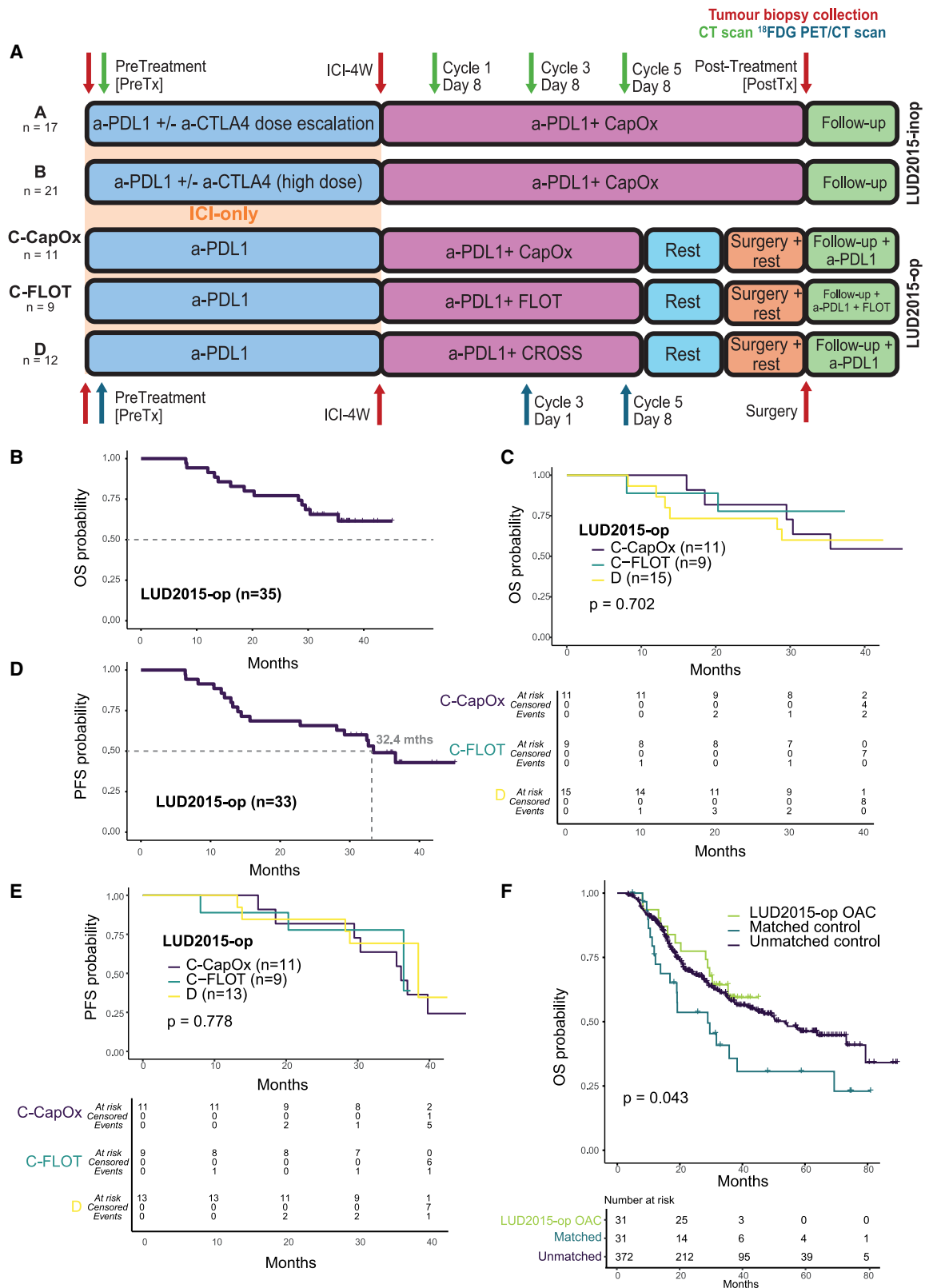
Immune checkpoint inhibitor (ICI) immunotherapy has become standard-of-care treatment for a range of cancer types, including metastatic esophageal cancer (oesophageal cancer, OC). Response to immunochemotherapy (ICI+CTX) is heterogeneous, and there is an unmet need for effective biomarkers to predict response. Programmed death-ligand 1 (PD-L1) expression¹ and microsatellite instability^{2,3} (MSI) are the only ICI biomarkers currently used in clinical practice. However, the clinical-utility thresholds of PD-L1 expression is arbitrary. Some tumors meet the thresholds yet do not respond to ICI/ICI+CTX,^{4–7} whereas PD-L1 low tumors can also benefit.^{8–10} While patients carrying MSI tumors tend to respond well to ICI-based therapies, MSI is infrequent in many cancers. Thus, it remains a priority to identify new biomarkers that can be implemented in the clinic to advance our ability to predict patient response to ICI/ICI+CTX, and ideally, such biomarkers should be applicable to both advanced and operable tumors.

OC has some of the poorest outcomes among cancer types, with the five-year overall survival (OS) rate remaining <15%. Since 2021, following the Food and Drug Administration (FDA) approval, ICI+CTX has become standard of care for patients with advanced OC and whose tumors have high PD-L1 expression. However, the prognostic value of existing biomarkers remains unsatisfactory: MSI is uncommon in OC,^{11,12} and CD8+T cell predictive value is mixed at best.^{13–15} Results from three large phase 3 trials in operable OC have been mixed. Checkmate-577 used adjuvant ICI after neoadjuvant chemoradiotherapy and surgery and showed a disease-free survival advantage, but indications for this approach are increasingly rare.¹⁶ KEYNOTE-585 used neoadjuvant pembrolizumab and did not meet the predefined threshold for statistical significance for event-free or OS, but most patients had a chemotherapy backbone of cisplatin and 5-fluorouracil (5-FU), which is no longer standard of care.¹⁷ Finally, MATTERHORN showed improvement in event-free survival with perioperative FLOT and ICI, which has led to FDA and European Medicines Agency (EMA) approval, and an anticipated change in the standard of care for oesophagogastric adenocarcinoma.¹⁸ All these emphasized the urgency and clinical need to identify clinically imple-

mentable biomarker and tumor subtyping capabilities to address who will benefit most from ICI-based combination therapy among operable OC, and to understand when and why it does not work.

The LUD2015-005 trial (NCT02735239) is a window-of-opportunity clinical trial that treated 38 patients with advanced/metastatic and inoperable OC (referred to herein as inoperable; LUD2015-inop) and 35 patients with operable OC with curative intent (LUD2015-op) using ICI+CTX and immunochemoradiotherapy (ICI+CRTX). ICI is routinely combined with standard-of-care treatment, but this trial was designed to include a 4-week ICI priming window (ICI-only) before combination with standard of care, thereby enabling scrutiny of the molecular effects of ICI both in isolation and in a combined preoperative treatment context. Through deep molecular profiling of the LUD2015-inop cohorts, we showed that tumor shrinkage was associated with an RNA-defined T/NK cell signature (INCITE) during ICI-only¹⁹ treatment. We also revealed that an increased tumor monocyte content (TMC) was associated with durable OS, and an increased incidence of >1 year progression-free survival, denoted as “clinical benefit” (CB).¹⁹ This unexpected finding raised the question of whether TMC also predicts good outcome in ICI-treated operable OC and whether this is applicable beyond upper gastrointestinal cancers.

Chemotherapy and targeted therapy aim to selectively kill cancer cells, whereas ICI-based therapy aims to alter the tissue/tumor immune microenvironment (TiME) to suppress tumor growth. Thus, to understand the molecular bases of heterogeneous patient response to ICI-based combination therapy, ICI+CTX or ICI+CRTX, we need a molecular readout that measures TiME. The TiME contains stromal cells, such as fibroblasts and endothelial cells, and tumor-infiltrating immune cells including innate (myeloid cells, monocytes, macrophages, neutrophils, etc.) and adaptive immune cells (T and B cells), which jointly make up the TiME. Tumors acquire distinct and widespread DNA methylation changes compared to their healthy surrounding tissue. In addition to DNA methylation changes within cancer cells, the different immune and stromal cells present in a tumor also feature unique methylomes. A study by Loyfer et al. showed that whole-genome methylation can capture cell composition features, suggesting that the DNA methylome of a



(legend on next page)

given tissue/tumor could potentially be used as a marker to characterize the TIME.²⁰ Building on our previous work that identified TMC as a powerful predictive biomarker of response to ICI-based combination therapy, using a single-cell RNA sequencing (scRNA-seq)-derived atlas to deconvolve tumor-derived bulk RNA-seq data, we hypothesize that the tumor DNA methylome (TDM) might identify distinct subtypes of OC with characteristic TIME. The TIME composition defined by TDM strengthened by the bulk RNA-seq derived from the same tumor biopsy can be compared to the adjacent normal tissues derived from the same patient, where possible.

Here, we report long-term survival data for patients undergoing curative intent treatment in the LUD2015-op trial arm and show that TMC is highly predictive of major pathological response (mPR; defined as Mandard 1 and 2) and OS. Combined whole-genome and DNA methylome with single-cell transcriptomic and bulk RNA-seq datasets generated from both LUD2015-inop and LUD2015-op cohorts revealed the impact of TMC on T cell function and the fate of tumor monocytes in the TIME and identified a TMC-related inflammatory myeloid minimal gene set CFISH (CXCL8, FCN1, IFI30, SLC11A1, and HLA-DRA) whose expression levels act as a clinically implementable biomarker for predicting OS regardless of tumor stage. Furthermore, we showed that tissue/tumor DNA methylomes are indirect readouts that classify TIME and identified five DNA methylation subtypes with varied clinical benefits, advancing our understanding of the molecular basis of heterogeneous patient response to ICI-based combination therapy.

RESULTS

A phase 2 immune-checkpoint-inhibitor-based neoadjuvant combination therapy trial of operable esophageal cancers reveals improved overall outcomes

The LUD2015-005 trial is a phase 1/2 ICI-based trial in which patients were given 4-week ICI priming with durvalumab, an anti-PD-L1 antibody (a-PDL1), followed by combination with standard-of-care therapy, either with palliative intent ($n = 38$, LUD2015-inop¹⁹) or with operable curative intent. The CONSORT study flowchart summarizes the recruitment for the LUD2015-op arm (Figure S1A). In total, 35 OC patients were enrolled to LUD2015-op study (Figure 1A). During the trial, new standard-of-care chemotherapy regimes were introduced in the UK. Thus, all LUD2015-op patients received a-PDL1

treatment and were divided into three cohorts depending on the standard of care received: neoadjuvant CapOx for cohort C-CapOx ($n = 11$), neoadjuvant FLOT²¹ for cohort C-FLOT ($n = 9$), and CRTX according to the CROSS regime²² for cohort D ($n = 15$). Patients were assigned to chemotherapy (CTX) or chemoradiotherapy (CRTX) treatment arms in multidisciplinary team meetings. Patients in cohort C-FLOT could also receive post-operative FLOT, and all patients could receive adjuvant ICI for up to 12 cycles. Among LUD2015-op, the median age was 60 years (range 33–72 years), and 88.6% (31/35) of patients were male; 82.9% (29/35) of patients had a cT3 or cT4a tumor and 80% (28/35) had nodal disease; 88.5% (31/35) of patient tumors were of adenocarcinoma histology (Tables S1, S2, and S3). Patients were staged with computed tomography (CT), ¹⁸FDG PET/CT scans, and laparoscopy, where clinically indicated.

Overall, treatment was well tolerated, and the study met its primary endpoint of safety and feasibility; 97.5% (156/160) of planned preoperative immunotherapy doses were given, with no new safety signals identified. All LUD2015-op patients experienced treatment-emergent adverse events (TEAEs), with 77.1% as grade 3 or above (27/35). Durvalumab-related events occurred in 77.1%, with 14.3% of these events at grade 3 or above. No LUD2015-op patient discontinued treatment due to a TEAE (Table S4). In LUD2015-op patients, treatment-emergent serious adverse events (TESAEs) occurred in 65.7% of patients (23/35) and were consistent with the known toxicities of the treatment components (Table S4B).

Two initially operable patients had progressive disease on CRTX and did not proceed to surgery. Of the 33 patients who received surgery, R⁰ resection was achieved in 81.8% (27/33). Anastomotic leaks were recorded in 6.1% (2/33). There were no perioperative deaths. mPR was seen in 48.5% (16/33) and complete pathological response in 24.2% (8/33). Restaging with ¹⁸FDG PET/CT after neoadjuvant therapy, available for 30 patients, identified complete and partial metabolic PERCIST responses in 20% (6/30) and 50% (15/30) of patients. irRECIST was evaluable in 32 patients, with complete and partial responses in 6.25% (2/32) and 18.75% (6/32), respectively (Tables S1 and S3). One patient in cohort D had a complete radiological response following ICI-only, with subsequent confirmed complete pathological response on resection. Two LUD2015-op patients with complete PERCIST response had tumors showing evidence of MSI.

Figure 1. A phase 2 immune-checkpoint-inhibitor-based neoadjuvant combination therapy trial of operable oesophageal cancers reveals improved overall outcomes in patients with adenocarcinoma

(A) Timeline showing the chronology of different treatment, sampling, and CT response assessment events during the LUD2015-005 trial for the four cohorts A–D, with A and B representing the advanced and C-CapOx/C-FLOT and D the operable cohorts. a-CTLA4 signifies the anti-CTLA4 treatment tremelimumab; a-PDL1 signifies durvalumab; CapOx signifies oxaliplatin and capecitabine; FLOT signifies 5-fluorouracil, leucovorin, oxaliplatin, docetaxel, every 2 weeks for 2 months²¹; CROSS signified paclitaxel and carboplatin once weekly along with daily radiotherapy for 5 weeks according to the CROSS regime.²² (B and C) Kaplan-Meier curves showing overall survival for patients with (B) operable esophageal adenocarcinoma (OAC, $n = 35$), (C) separated by neoadjuvant treatment arm.

(D and E) Kaplan-Meier curves showing progression-free survival (PFS) for patients with (D) operable OC who proceeded to surgery ($n = 33$ total; C-CapOx, $n = 11$; C-FLOT, $n = 9$; D, $n = 10$; D2, $n = 3$), (E) separated by neoadjuvant treatment arm.

(F) Kaplan-Meier curves showing survival probabilities for OAC patients treated in LUD2015-op, compared to patients treated over the same period with CapOx, CROSS, or FLOT but not ICI (“control”). Both unmatched (blue) and propensity-score matched (teal) patients are compared to LUD2015-005 survival outcomes. Propensity score matching variables were age, comorbidities, tumor N stage, and treatment protocol. p value is from log rank test between LUD2015-op OAC patients and matched control patients.

With a minimum follow-up of 2 years, median OS was not reached for any of the three cohorts (Figures 1B and 1C), and median progression-free survival was 32.4 months (Figures 1D and 1E). Overall LUD2015-op 2-year and 30-month survival rates were 77.1% and 68.6% on an intention-to-treat basis, respectively. 2-year and 30-month survival rates for cohort C-CapOx ($n = 11$) were 81.8% and 72.7%, for the C-FLOT arm ($n = 9$) 77.8% and 77.8%, and for cohort D ($n = 15$) 73.3% and 60%, respectively. After propensity score matching with 403 patients treated for operable esophageal adenocarcinoma (oesophageal adenocarcinoma, OAC) without immunotherapy in Oxford, the main trial center, using the same neoadjuvant therapies as in LUD2015-op, we observed that median survival in the matched cohort is 28.8 months (95% confidence interval [CI] 16.8 months–NA) and not reached (95% CI 30.4 months–NA) in the LUD2015-op trial. These results suggest that patients in LUD2015-op had improved survival when compared to patients receiving standard of care without immunotherapy ($p = 0.043$, Figures 1F and S1B–S1D).

Tumor monocyte content, but not infiltrating CD8⁺ T cells, predicts good survival of neoadjuvant immunochemotherapy-treated operable esophageal cancer patients

To understand the molecular basis of heterogenous patient response to ICI-based therapy, we took advantage of the unique design of our trial, which collected longitudinal biopsy samples from multiple sites. Samples were collected from tumor, normal esophagus, and gastric tissues at the following time points: before treatment (“PreTx”), 4 weeks post ICI-only (“ICI-4W”), and after completion of ICI+CTX or ICI+CRTX treatment (Figure S2A). A detailed analysis of paired sample sets from PreTx and ICI-4W allowed us to investigate how a given patient’s tumor and normal tissues respond to ICI-4W treatment.

To investigate what underlies heterogenous patient response to ICI+CTX, scRNA-seq was performed on primary tumor biopsies of 16 LUD2015-op patients where suitable tissue was available for all time points, of whom 10 patients achieved mPR. These data were integrated with scRNA-seq data from LUD2015-inop, to yield a pan-stage OC scRNA-seq atlas comprising 117,874 cells that consists of 52 cell types across 11 cell compartments (Figure 2A). This newly generated scRNA-seq atlas showed that immune cells derived from inoperable or operable OCs and from tumors of different histologies or different stages have similar transcriptomes (Figure S2B). However, some immune cell types were relatively more abundant in certain tumor samples (Figure S2C). Cell-type annotation is illustrated in Figure S2D.

ICI-based therapies rely on tumor-infiltrating CD8⁺ T cells. A subset of CD8⁺ tissue resident memory (CD8⁺ TRM) T cells in particular is likely to be tumor-reactive, characterized by CD39/CD103 expression^{23–26} (Figure S2E). Thus, the scRNA-seq atlas was used to deconvolute paired bulk RNA-seq data using BayesPrism²⁷ as previously described¹⁹ to estimate the abundance of tumor-infiltrating CD8⁺ TRM cells in 25 PreTx/ICI-4W-paired LUD2015-op samples and observed that CD8⁺ TRM infiltration during ICI-only is highly variable and only observed in some samples (Figure 2B). In contrast, CD8⁺ TRM

infiltration is observed in most LUD2015-inop patients (Figure S2F). These findings suggest that advanced tumors are more likely to upregulate previously absent CD8⁺ TRM in response to ICI-only, whereas some operable patients tend to have high levels of CD8⁺ TRM PreTx (Figure S2G) that are not further upregulated by ICI-only. CD8⁺ TRM cell frequency in PreTx LUD2015-op samples does not correlate with patient OS (Figure S2H). This indicates that both patients with and without abundant T and NK cells at PreTx can benefit from neoadjuvant ICI+CTX/CRTX.

Hierarchical clustering reveals instead that LUD2015-op patients with high myeloid cell content at PreTx were significantly more likely to be alive at the end of the study ($p = 0.007$) (Figure 2C), illustrating a strong link between innate immune composition and OS in ICI+CTX/CRTX-treated LUD2015-op. Specifically, a high TMC at PreTx, as determined by deconvolution, was highly predictive of OS (Figure 2D). This mirrors observations in LUD2015-inop,¹⁹ posing TMC as a powerful predictor of ICI-treated OC survival regardless of tumor stage and operability (Figure S2I).

The expression levels of a TMC-related five-gene signature “CFISH” (CXCL8, FCN1, IFI30, SLC11A1, and HLA-DRA) predict response and overall survival to immunochemotherapy

To explore the clinical application potential of TMC in stratifying patients for ICI-based combination therapy, we aimed to identify the minimum number of genes that can serve as a proxy for TMC. To achieve this, the 30 most enriched monocyte marker genes were extracted from the scRNA-seq atlas (Figure 3A). Using linear modeling and best subset regression, a five-gene signature (CXCL8, FCN1, IFI30, SLC11A1, and HLA-DRA), CFISH, emerged as the best predictor of deconvoluted TMC (Figures S3A–S3C). CFISH performs similarly to TMC: PreTx CFISH expression is significantly higher in patients with mPR and prognostic of long OS in LUD2015-op (Figures 3B and 3C) and as a pan-stage OS predictor (Figure 3D) when patients are split by CFISH median. As a continuous variable, CFISH also predicts survival (C-index TMC 0.62, CFISH 0.61; Figure S3D).

CFISH weakly correlates with tumor mutational burden (TMB) (Figure S3E), but the TMB-high CFISH-high patient group, split by median CFISH and TMB, respectively, shows the longest survival (Figure S3F; multivariate Cox regression: CFISH $p = 0.049$, TMB $p = 0.065$), showing that the two biomarkers are independent). Notably, CFISH expression was not prognostic for OS for CTX-treated OC patients from the International Cancer Genome Consortium³² (Figure S3G), underlining that the prognostic value of CFISH, like TMC,¹⁹ is specific to ICI-based combination therapy.

The predictive potential of CFISH in other ICI+CTX-treated cancer types was also validated in RNA-seq datasets derived from cohorts of ICI+CTX-treated gastric cancer, melanoma, and non-small cell lung cancer (NSCLC): the expression levels of CFISH were significantly higher in RECIST responders (Figure 3E), outperforming a range of previously described ICI biomarkers (Figure S3H), suggesting that CFISH may have prognostic value in cancers of different tissues of origin treated with ICI-based therapy. Circulating monocyte count has been linked

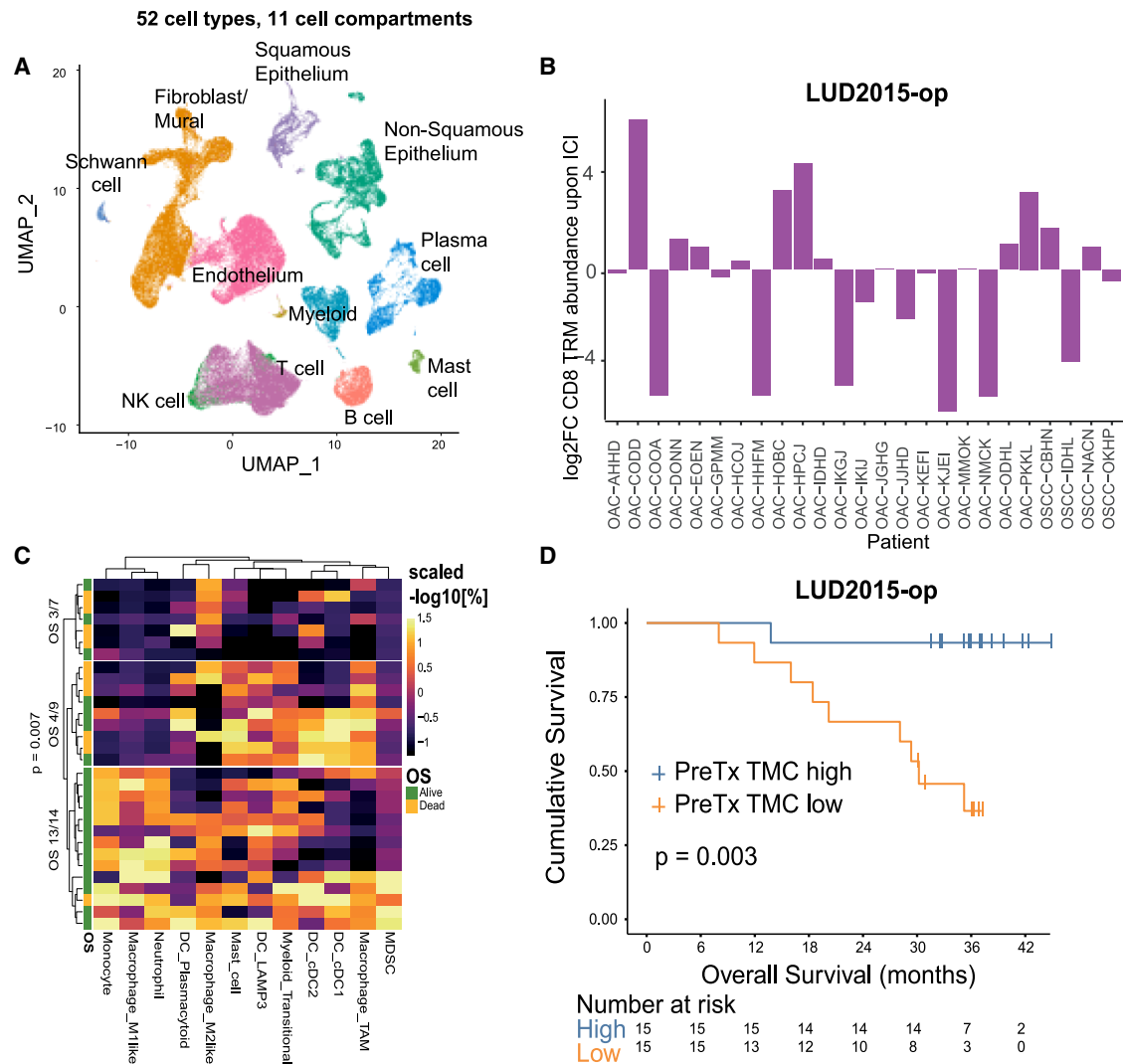


Figure 2. Tumor monocyte content, but not infiltrating CD8⁺ T cells, predicts good survival of neoadjuvant ICI+CTX/ICI+CRTX-treated operable OC patients

(A) UMAP dimensionality reduction of scRNA-seq compartments encompassing samples from LUD2015-op, LUD2015-inop, and four patients with Barrett's esophagus. Cells from 52 cell types are clustered into 11 color-coded compartments.

(B) Bar chart showing degree of CD8⁺ TRM infiltration upon ICI in operable OC samples, displayed as log₂ fold change in proportion of all cells, as determined by deconvolution.

(C) Deconvolution-assessed levels of myeloid cell types as a proportion of total cells in LUD2015-op PreTx OAC biopsies were log₁₀-transformed and scaled by column prior to hierarchical clustering (ward.D linkage). Results are shown as a clustered heatmap, with cells colored according to the scaled deconvolution estimates and row annotation according to clinical outcomes (mPR and overall survival). mPR and OS rates are shown for the clusters as indicated by the vertical lines. *p* value (for difference between these rates) by Fisher's exact test.

(D) Kaplan-Meier plot showing OS of PreTx TMC-high and -low groups from LUD2015-op patients, split by the cohort median. *p* value by log-rank test.

to poor ICI response,³³ but TMC and CFISH expression show no strong link with blood monocyte levels (Figure S3I), which indeed are not correlated with long survival in LUD2015-005 (Figure S3J). Finally, we used qPCR to validate the CFISH score derived from RNA-seq estimation. qPCR for the expression levels of CFISH were carried out using the bulk RNA extracted from PreTx tumor samples from 12 patients used in the above-mentioned RNA-seq with estimated low, medium, and high CFISH expression scores (four patients per group). The results

shown in Figure 3F confirms that the CFISH score correlates very strongly with CFISH gene expression. CFISH genes were identified based on their predictive value for TMC, but CFISH genes are not exclusively expressed in monocytes (Figures S3J and S3K), so the CFISH gene signature may capture a broader inflammatory myeloid TME. Four gene CFISH subsets are also predictive of survival as long as FCN1 and SLC11A1 are included, which are not prevalent in M2-like and tumor-associated macrophages (Figure S3L). As a continuous variable,

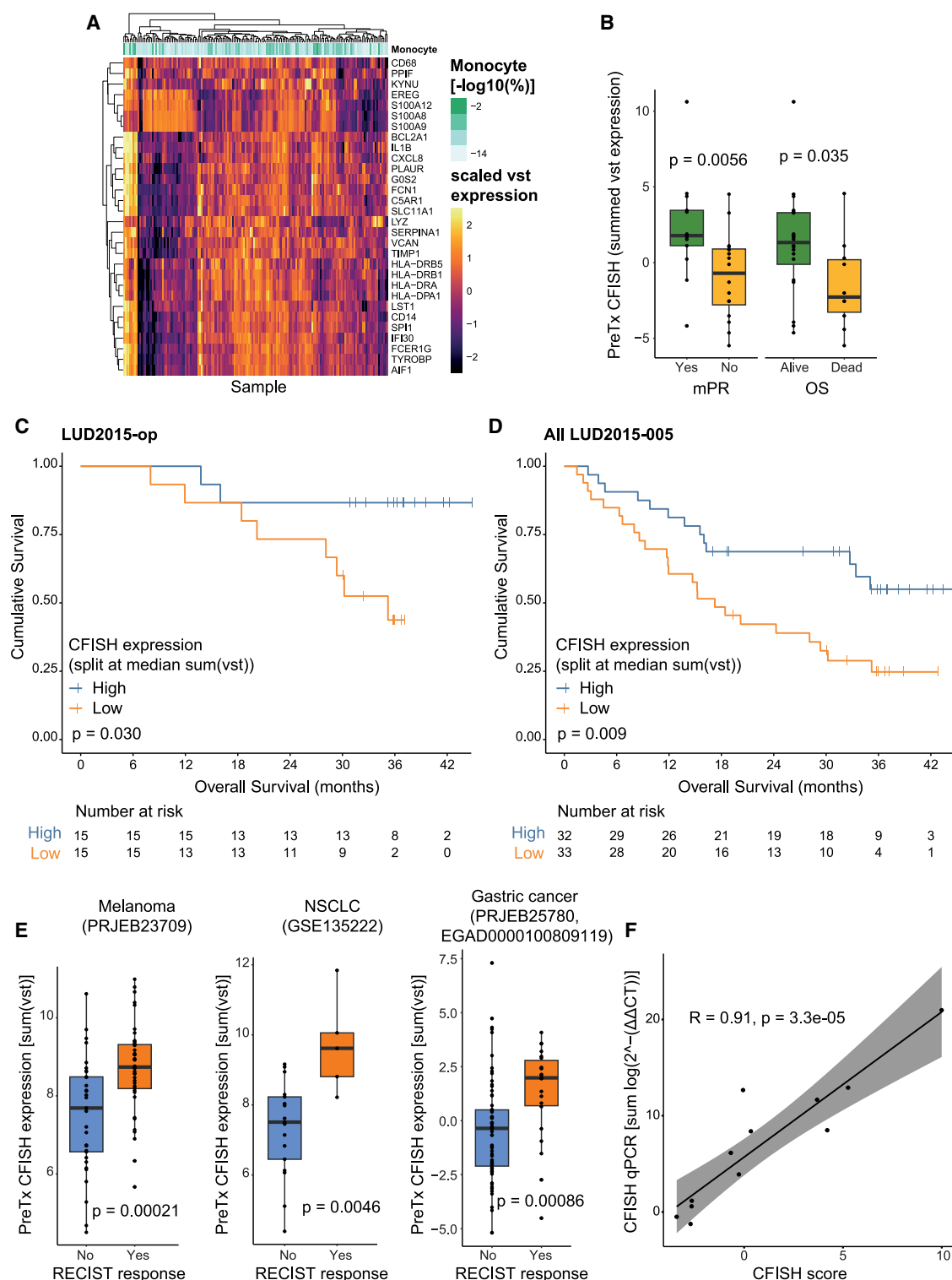


Figure 3. The expression levels of a TMC-related gene signature “CFISH” (CXCL8, FCN1, IFI30, SLC11A1, and HLA-DRA) predict response and overall survival to immunochemotherapy

(A) Expression of top 30 monocyte marker genes. Monocyte markers were identified by the FindMarkers function of Seurat, comparing clusters annotated as monocytes with all other cell types. logTPM expression values were scaled before hierarchical clustering (ward.D), and results are shown as a clustered heatmap with cells annotated according to scaled expression value. Columns are annotated by $-\log_{10}$ -transformed TMC as estimated by deconvolution.

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CFISH also predicts survival, and direct comparisons of TMC and CFISH at 1, 2, and 3 years showed similar discriminatory ability, suggesting that CFISH is representative of TMC (C-index TMC 0.62, CFISH 0.61; [Figure S3D](#)).

All these demonstrated that CFISH is a valuable and clinically implementable biomarker that predicts patient response and OS to ICI+CTX.

Tumor monocytes contribute to tumor immune microenvironment remodeling and associate with tumor-reactive CD8⁺ T cells upon immunochemotherapy treatment

To understand the biology underlying the prognostic value of high TMC, we hypothesized that monocytes may be the source of a cytotoxic T cell-supportive myeloid niche. High TMC only at PreTx, but not at later time points, predicts survival ([Figure S4A](#)), and monocytes are known to differentiate into effector cells upon tissue entry.^{34,35} Therefore, the cell types into which they differentiate may influence the TiME and CD8⁺ T cell function. While we showed previously that the total CD8⁺ T cell numbers show no relationship with survival in this, the proportion of tumor-reactive CD8⁺ T cells might associate with survival. We observed that the frequency of CD39/CD103 CD8⁺ T cells, exhibiting a tumor response phenotype,²³ increased specifically in patients with mPR (LUD2015-op) and CB (LUD2015-inop) at ICI-4W ([Figure 4A](#)), suggesting a link between the proportion of tumor-reactive CD8⁺ T cells and OC patient response to ICI+CTX/CRTX. Notably, immune response enrichment analysis³⁶ shows that CD39/CD103 CD8⁺ T cell transcriptomes exhibit enrichment of interleukin-2 (IL-2) and (IL-15) signaling ([Figure 4B](#)). Beyond low-level IL-15 expression in some endothelial cells, myeloid cells, specifically M1-like macrophages and LAMP3-high dendritic cells (DCs), are the main source of IL-15 in the TiME ([Figures S4B–S4E](#)). Monocytes upon tissue entry can differentiate into macrophages and DCs with a cDC2-like phenotype,³⁷ and we hypothesized that tumor monocytes might remodel the myeloid compartment and CD8⁺ T cell function.

To test this, we carried out single-cell trajectory and RNA velocity analysis on the myeloid compartment from the pan-OC single-cell atlas containing cells from 88 biopsies to map monocyte fate. The results in [Figure 4C](#) demonstrate that monocytes are likely to differentiate along two trajectories: the first toward pro-inflammatory M1-like macrophages and the second toward cDC2 and LAMP3hi DC. Linear mixed effect modeling, which lends itself to time series analyses with fixed effects (monocyte

abundance and time point) and random effects (patient ID), reveals that monocytes associate with LAMP3hi DC and M1-like macrophage levels and different T cell types ([Figure 4D](#)). Conversely, monocytes negatively associate with the abundance of tumor cells and related deconvolution signatures (columnar-like and proliferative epithelium) ([Figure 4D](#); [Table S7](#)). Further, in patients with high PreTx TMC, there is an increase in the ratio of CXCL9 to SPP1 expression, markers of inflammatory T cell-supporting macrophages that associate with ICI response³⁸ ([Figure 4E](#)).

To investigate those features in the TiME accompanying monocyte differentiation, gene expression modules that changed along differentiation trajectories were identified ([Figures S4F and S7G](#); [Table S8](#)). Immune response enrichment analysis of genes included in M1-like macrophage and LAMP3hi DC-associated gene modules suggests that monocyte to M1-like macrophage and LAMP3hi DC differentiation is accompanied by interferon (IFN) and IL-15 signaling ([Figure 4F](#)), suggesting they are not only sources of IL-15 but are also induced by IL-15, representing a feedforward loop. IL-15 and IFN also emerge as the most enriched cytokine signaling pathways among genes highly expressed in patients with mPR at ICI-4W ([Figure 4G](#)). IL-15 and IFN signaling at ICI-4W is highly correlated with the abundance of cDCs, M1-like macrophages, and differentiating DCs at ICI-4W ([Figure 4H](#)).

Finally, as LAMP3hi DC supports anti-tumor T cell function in the context of ICI+CTX,^{38–43} it is reassuring that CD39 and CD103 expression among CD8⁺ T cells is higher in patients with high levels of LAMP3hi DC at ICI-4W ([Figure S4H](#)). Cell-cell communication analysis using NicheNet confirms that monocyte-derived LAMP3-high DCs are the main senders of ligands for the most enriched receptors expressed by tissue-resident CD8⁺ T cells ([Figure S4I](#)). This suggests that tumor monocytes contribute to TiME remodeling and may positively influence tumor-reactive CD8⁺ T cell function upon ICI+CTX/CRTX.

INCITE is a readout of tumor immune microenvironment heterogeneity and dynamics in response to ICI treatment

Our previous study in the inoperable setting showed that ICI-only induces widespread transcriptomic changes among tumor samples, the most notable of these changes being the upregulation of a T/natural killer (NK) cell inflammatory gene signature, coined INCITE.¹⁹ Building on this, in this study, we examined ICI-induced transcriptomic changes in 25 LUD2015-op patients

(B) (Left) Boxplots comparing the PreTx five-gene monocyte gene expression score between operable OC patients alive and deceased at the end of the study and (Right) patients with and without mPR at surgery. Expression score is calculated from the sum of z-scaled mean VST-normalized expression. *p* value by Wilcoxon test.

(C) Kaplan-Meier plot showing OS of PreTx CFISH high and low groups from operable OC patients, split by median summed VST-normalized expression of CFISH genes.

(D) Kaplan-Meier plot showing OS of PreTx CFISH high and low groups from all LUD2015-005 patients, split by median summed VST-normalized expression of CFISH genes. *p* value by log-rank test.

(E) Boxplots comparing PreTx five-gene monocyte gene expression score (CFISH) between ICI-treated gastric cancer patients with and without response according to RECIST/RECIST v.1.1 from a published RNAseq dataset^{28,29} and from published ICI-treated melanoma³⁰ and NSCLC³¹ cohorts. *p* value by Wilcoxon test.

(F) Scatterplot showing the correlation of CFISH score (summed VST-normalized expression of CFISH genes, *x* axis) and CFISH expression by qPCR [summed log-transformed 2^{-ΔΔCT}]. qPCR was performed in triple repeats from PreTx tumor samples from 12 patients from LUD2015-op, selected based on low, medium, and high levels of CFISH expression (four patients per group). Correlation coefficient and *p* value by Pearson test.

with available paired PreTx and ICI-4W tumor biopsies. We also performed bulk RNA-seq analysis of normal gastrointestinal tissues (squamous esophagus) in a subset of patients: we selected five patients from each group, including patients with and without CB (LUD2015-inop) and mPR (LUD2015-op). Additionally, we performed RNA-seq on normal gastric tissue from five LUD2015-inop patients.

Strikingly, we observed little change in gene expression in tumor samples upon ICI-only in LUD2015-op: only one gene was differentially expressed, mirroring the low number of differentially expressed genes in examined normal tissues. This contrasts with LUD2015-inop, where, among all tumor biopsies, ICI-only upregulated 391 and downregulated 75 genes (Figure 5A). However, when comparing normal tissues and corresponding tumors from the same patients, we found that normal tissues of the same type cluster together and distinctly from tumor samples and that transcriptomes from LUD2015-op tumor biopsy samples are particularly heterogeneous (Figure 5B). This suggests that ICI-induced transcriptomic changes depend on PreTx tissue characteristics, such as tumor TiME composition.

INCITE gene expression at PreTx (INCITE(PreTx)) was variable among LUD2015-op tumors (Figure 5C), which can be divided into INCITE(PreTx)-high and INCITE(PreTx)-low tumors. These groups show distinct transcriptomic changes in response to ICI-only (Figure 5D). Detailed examination of differentially expressed genes upon ICI-only revealed that 7 of 21 upregulated genes among INCITE(PreTx)-low LUD2015-op samples are also upregulated in LUD2015-inop, including four INCITE genes, while none were shared with INCITE(PreTx)-high LUD2015-op samples. This suggests that LUD2015-op biopsies with low INCITE(PreTx) expression, as LUD2015-inop, may acquire INCITE gene expression upon ICI-only treatment.

The variable ICI-induced transcriptomic changes depend on PreTx tissue features and suggest that INCITE(PreTx) levels may be linked to TiME composition. We estimated the cellular composition of 55 paired PreTx and ICI-4W tumor samples (30 LUD2015-inop and 25 LUD2015-op tumor samples) and 10 paired PreTx and ICI-4W normal esophagus samples using deconvolution to investigate whether a heterogeneous TiME composition underlies the observed variable transcriptomic response to ICI-only treatment.^{19,27} As expected, little to no immune cells are detected in 20 paired normal esophagus, both at PreTx and ICI-4W (Figure S5A), consistent with few ICI-only-induced transcriptomic changes in all normal tissues examined (Figure 5A). In 55 paired tumor samples, however, immune infiltration is readily detectable, with variable abundance of myeloid, T and NK, B, and plasma cells (Figure 5E). Consistent with this, INCITE genes are also most highly expressed in NK and T cells (Figure S5B).

LUD2015-op tumors show heterogeneous PreTx immune composition, and hierarchical clustering of patient TiME composition splits LUD2015-op samples into two groups, overlapping with INCITE(PreTx) expression (Figure S5C). Compared to LUD2015-op INCITE(PreTx)-low and LUD2015-inop PreTx tumors, LUD2015-op INCITE(PreTx)-high tumors show higher infiltration of CD8⁺ TRM cells, NK cells, immunoglobulin G (IgG) plasma cells, and cDC1s at PreTx (Figures 5F and S5D). The increased PreTx immune abundance is paralleled by increased capillary endothelial frequency, indicative of rich vascular supply to INCITE(PreTx)-high tumors (Figure S5E). Differential gene expression upon ICI-4W is accompanied by TiME composition changes: in INCITE(PreTx)-low tumors, but not INCITE(PreTx)-high tumors, INCITE-expressing T and NK cells and DCs increase in abundance upon ICI-only treatment (Figure 5G). At ICI-4W, there is no difference in abundance in cDC, IgG

Figure 4. Tumor monocytes contribute to tumor immune microenvironment myeloid compartment remodeling and associate with tumor-reactive CD8⁺ T cells upon immunochemo(radio)therapy

(A) Bar plot showing median normalized proportion of CD39- and CD103-expressing cells among CD8⁺ T cells derived from each LUD2015-005 patient, comparing patients with and without CB (LUD2015-inop) or mPR (LUD2015-op) at PreTx and ICI-4W. *p* value by Wilcoxon.

(B) Bar charts showing the 10 most enriched cytokine signaling pathways among genes significantly more highly expressed in CD39/CD103 double-positive CD8⁺ T cells compared to single-positive/double-negative CD8⁺ T cells, as determined by immune response enrichment analysis. Bar length represents the enrichment score, and color reflects the adjusted *p* value, which is also labeled.

(C) (Left) UMAP generated by monocle3 analysis, where myeloid compartment cells are ordered by pseudotime along differentiation trajectories also calculated by monocle3, with cells color-coded by cell subtype. (Middle) UMAP dimensionality reduction of the scRNA-seq myeloid compartment. Cells are color-coded by myeloid cell subtype. (Right) Differentiation direction vectors identified from RNA velocity analysis with velocraptor are overlaid onto the same UMAP shown in the middle panel. Overarching differentiation trajectories are manually superimposed in orange.

(D) Dot plot showing the association of log10-transformed monocyte levels, as calculated by deconvolution, with the abundance of other cell types in a linear mixed effect model constructed with monocytes and time points (relative to PreTx) as fixed effects and patient ID as random effect. The left panel shows cell types with a positive association, and the right panel shows those with a negative association. Dot size reflects the effect size, and color reflects the *p* value from the Satterthwaite degrees of freedom approximation. All cell types with a *p* value <0.1 are shown.

(E) Boxplots showing the ratio of CXLC9 and SPP1 expression in bulk transcriptomic data, calculated from logTPM, comparing PreTx and ICI-4W expression ratios. TMC-high and -low patients are compared, split by median VST-normalized summed expression of monocyte five-gene signature CFISH. *p* value by Wilcoxon test.

(F) Bar charts showing the 10 most enriched cytokine signaling pathways by immune response enrichment analysis. Bar length represents the enrichment score, and color reflects the adjusted *p* value, which is also labeled. Immune response enrichment analysis results are shown for genes from (left) modules 13 and 16 in macrophages (right) and module 12 in DC. Results for IL-15 are outlined within a red box.

(G) Differential gene expression between operable OC patients at ICI-4W with and without mPR. (Left) Volcano plot shows moderated log fold change and false discovery rate (FDR). All significantly differentially expressed genes (DEGs) (FDR <0.1) are in red; the top 70 significant genes are labeled. (Right) Bar charts showing the 10 most enriched cytokine signaling pathways among genes significantly higher expressed in patients with mPR at ICI-4W by immune response enrichment analysis. Bar length represents the enrichment score, and color reflects the adjusted *p* value, which is also labeled.

(H) Scatterplot showing the correlation of expression of IL-15 and IFN response genes at ICI-4W, displayed as summed VST-normalized expression, and the abundance of M1-like macrophages, cDCs, and differentiating DCs at ICI-4W, as determined by deconvolution for all LUD2015-005 patients. Correlation coefficient and *p* value by Pearson test.

plasma cells, NK cells, and CD8⁺ TRM abundance between INCITE(PreTx)-low and INCITE(PreTx)-high operable OC (Figure S5F), suggesting that ICI-only treatment compensates for the differences in immune cell infiltration observed at PreTx.

Tumor cell malignancy and its metastatic potential is intimately linked to its TiME: a tumor-supportive TiME favors the metastasis of tumor cells to lymph nodes, and the extent of tumor cell malignancy and tumor promoting TiME associates with the presence of tumor cells in adjacent or distant lymph nodes. While immune-mediated tumor killing works in opposition to tumor progression, the extent of tumor visibility to the immune system is likely to correlate with the extent of tumor spread. As INCITE score can be used as an indirect readout of TiME dynamics, we therefore hypothesized that INCITE scores might be linked to tumor metastatic potential, in the context of therapies that activate the immune system. Both operable and inoperable OACs were grouped according to the sequential extent of cancer spread: local organ involvement only, peritumoral lymph node involvement, then distant lymph node involvement, and finally radiological evidence of solid organ metastasis. INCITE showed similar baseline values in all cancer states, but the induction of INCITE in response to ICI was associated with cancer spread based on lymph node status and shown to be significantly higher in more advanced cancer, demonstrated in a stepwise fashion (Figure S5G). Taken together, the INCITE score based on the differential changes to INCITE gene expression in response to ICI can act as an indirect readout of TiME and can provide further insight into TiME dynamics in response to ICI treatment.

Tumor DNA methylation classifies OAC into five subtypes

Each cell type features distinct DNA methylation patterns. The generation of a tissue DNA methylation atlas enabled the assessment of tumor-unique features as well as cell-type composition.²⁰ Meanwhile, robust approaches have been developed to deconvolve bulk RNA-seq data into cell-type components. As DNA methylation is linked to gene expression, the combination of tissue methylation data and bulk RNA-seq deconvolution-derived cell composition estimates provides an opportunity to both identify tumor subtypes by their distinct methylation features and characterize their unique cell-type composition. On this basis, the DNA methylome can then be utilized as a proxy readout to classify TiME.

To test this hypothesis, we performed unbiased whole-genome DNA methylation analysis of adjacent normal esoph-

agus, stomach, and duodenum tissues and 52 PreTx OAC tumor samples (23 and 29 LUD2015-inop and -op, respectively), excluding rare esophageal squamous cell carcinoma (oesophageal squamous cell carcinoma, OSCC) samples (which comprised only 7 OSCC/73 total OC in the LUD2015-005 cohort). Whole-genome DNA methylation was detected using our newly developed TET-assisted pyridine borane sequencing (TAPS) technology.^{11,12,44–50} Unlike bisulfite sequencing, TAPS directly reads modified cytosine, while preserving unmodified cytosine, and results in cleaner, more evenly covered data even from low amounts of input material, which is of particular benefit when dealing with small biopsies. To enable direct association of RNA-seq and TAPS data, each biopsy was bisected; one-half was processed for bulk RNA-seq (described above), while the remainder was used to extract DNA for whole-genome sequencing (WGS) and TAPS. To compare the data derived from two different DNA methylation sequencing platforms, we also included whole-genome bisulfite (WGBS)-derived data from two Barrett's esophagus samples, one high-grade esophageal dysplasia sample, and six adjacent normal samples from esophagus, stomach, and duodenum from the same two patients. We first identified 395,503 differentially methylated 1-kb regions (DMRs) between tumor and adjacent esophageal epithelium and performed unsupervised clustering on these for the entire LUD2015-005 trial OAC cohort (see STAR Methods). Notably, the WGBS adjacent normal controls cluster well with TAPS adjacent normal controls of the correspondent tissue type. The identified DMR classified PreTx samples into five distinct tumor DNA-methylation-derived tumor subtypes (TDM-TS): OAC with high (OAC-MethH), medium (OAC-methM), and low (OAC-methL) genome-wide methylation levels and two subgroups that closely resemble esophagus (OAC-oeso) and stomach/duodenum (OAC-SD), respectively (Figures 6A and 6B). We classified MSI tumors as a sixth subtype owing to their unique genomic and molecular features (Figure S6A). We confirmed that the observed five TDM-TS are robust and stable by repeatedly sub-sampling 55K regions from all 396K DMRs and repeating the clustering, then plotting the congruence of cluster assignment per sample: most samples are consistently clustered in the same groups irrespective of DMR subset (Figure S6B).

Tumor content per sample, estimated by copy-number analysis of WGS data (see STAR Methods), varies across samples but largely agrees with RNA-seq-derived purity estimates (Figures 6C and S6C–S6E). Previous work indicates that levels of chromosomal instability, defined by high rate of numerical or structural chromosome alterations, vary in gastro-oesophageal

- (B) PCA plots of bulk transcriptomic data from (upper) five advanced and five operable OC patients, encompassing tumor biopsies (LUD2015-inop and LUD2015-op) and matched normal biopsies (gastric and esophagus) from the same patients and (lower) from all PreTx samples. Points are color-coded by tissue of origin.
- (C) Bar chart showing the sum of mean VST expression of INCITE genes at PreTx for each patient of the operable cohort.
- (D) Volcano plots showing differential gene expression from PreTx to ICI-4W in INCITE-high (left) and -low (right) patients, split by median INCITE expression at PreTx. Significantly DEGs are colored in red and top 70 DEGs are labeled.
- (E) Bar charts showing deconvolution-assessed proportion of different cell compartments in advanced and operable OC samples. Each pair of bars represents tumor samples from a patient at PreTx (left) and ICI-4W (right).
- (F) Boxplots showing log₁₀ transformed PreTx abundance of cell types by deconvolution for LUD2015-inop patients compared to LUD2015-op INCITE(PreTx)-low and INCITE(PreTx)-high patients. *p* value by Kruskal Wallis test.
- (G) Boxplots showing log₂FC from PreTx to ICI-4W in abundance of dendritic cells (cDC 1, cDC2, LAMP3hi cDC), CD8⁺ tissue-resident memory T cells, NK cells, and IgG plasma cells from PreTx to ICI-4W in LUD2015 patients. Plots are faceted by PreTx INCITE expression, split at median of the sum of mean VST-normalized expression. *p* value by Wilcoxon test.

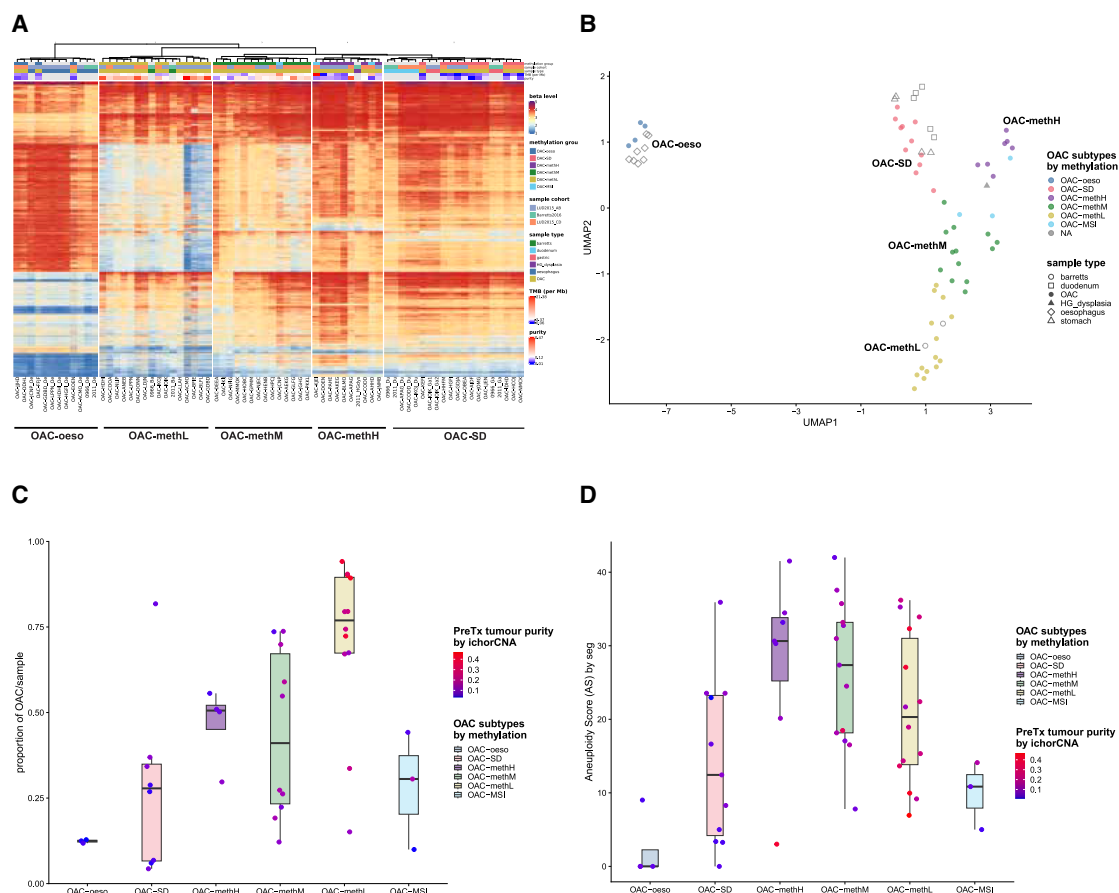


Figure 6. Tumor DNA methylation classifies OAC into five subtypes

(A) Heatmap of methylation values in 55K out of 396K DMR regions per PreTx tumor sample, together with healthy esophageal, gastric, and duodenal samples, as well as Barrett's esophagus and high-grade dysplasia samples. Hierarchical clustering shown as dendrogram, splitting all samples into five tumor DNA methylation-derived tumor subtypes (TDM-TS). Top annotations: methylation-derived tumor subtypes (with microsatellite unstable [MSI] highlighted in light blue), sample cohort, sample type, purity estimates (by ichorCNA), and TMB.

(B) UMAP projection using all 396K DMRs. Points are colored by TDM-TS, with point shape denoting sample type.

(C) Boxplot showing estimated OAC fraction per sample from RNA-seq deconvolution, grouped by five TDM-TS and MSI subtypes. Dots colored by tumor purity of the sample measured by ichorCNA.

(D) Boxplot showing large-scale genome instability per sample measured by aneuploidy score, grouped by five methylation subtypes and MSI subtype. Dots colored by tumor purity of the sample measured by ichorCNA.

cancers. Interestingly, we observe high aneuploidy scores in OAC-methH and OAC-methM, while OAC-methL and OAC-SD rank lower, and OAC-oeso and OAC-MSI (which are characterized by abundant small insertions and deletions and single-nucleotide variations) exhibit the lowest levels of chromosomal instability (Figure 6D). This pattern is unrelated to tumor purity, which is estimated based on DNA copy-number variations (Figure S6F), highlighting that DNA-methylation subtypes capture unique biological features in OAC.

Distinctive tumor DNA methylation subtypes stratify TIME and clinical response to immunochemotherapy

We showed above that RNA expression levels of INCITE genes, and the change in these gene expression levels between untreated and ICI-4W treated samples derived from the same patient, reflect TIME and its dynamics. If the identified five

tumor DNA-methylation subtypes can classify TIME, we expect to observe an association between DNA-methylation subtypes and the change of INCITE expression before and during treatment.

To test this, we used the bulk RNA-seq data to compare the INCITE level at PreTx and their change (INCITE score) upon ICI treatment among the identified five DNA-methylation subtypes. Notably, we observed a clear association between methylation subtype and INCITE score: OAC-oeso and OAC-SD exhibit no significant change in INCITE score upon treatment, unlike samples in the other clusters, despite similar INCITE score distribution at baseline, suggesting that these clusters feature distinct differences in their TIME (Figure 7A).

To investigate the potential of DNA-methylation subtypes to classify TIME in each cluster more thoroughly, we leveraged the 52 distinct cell-type contents that were previously

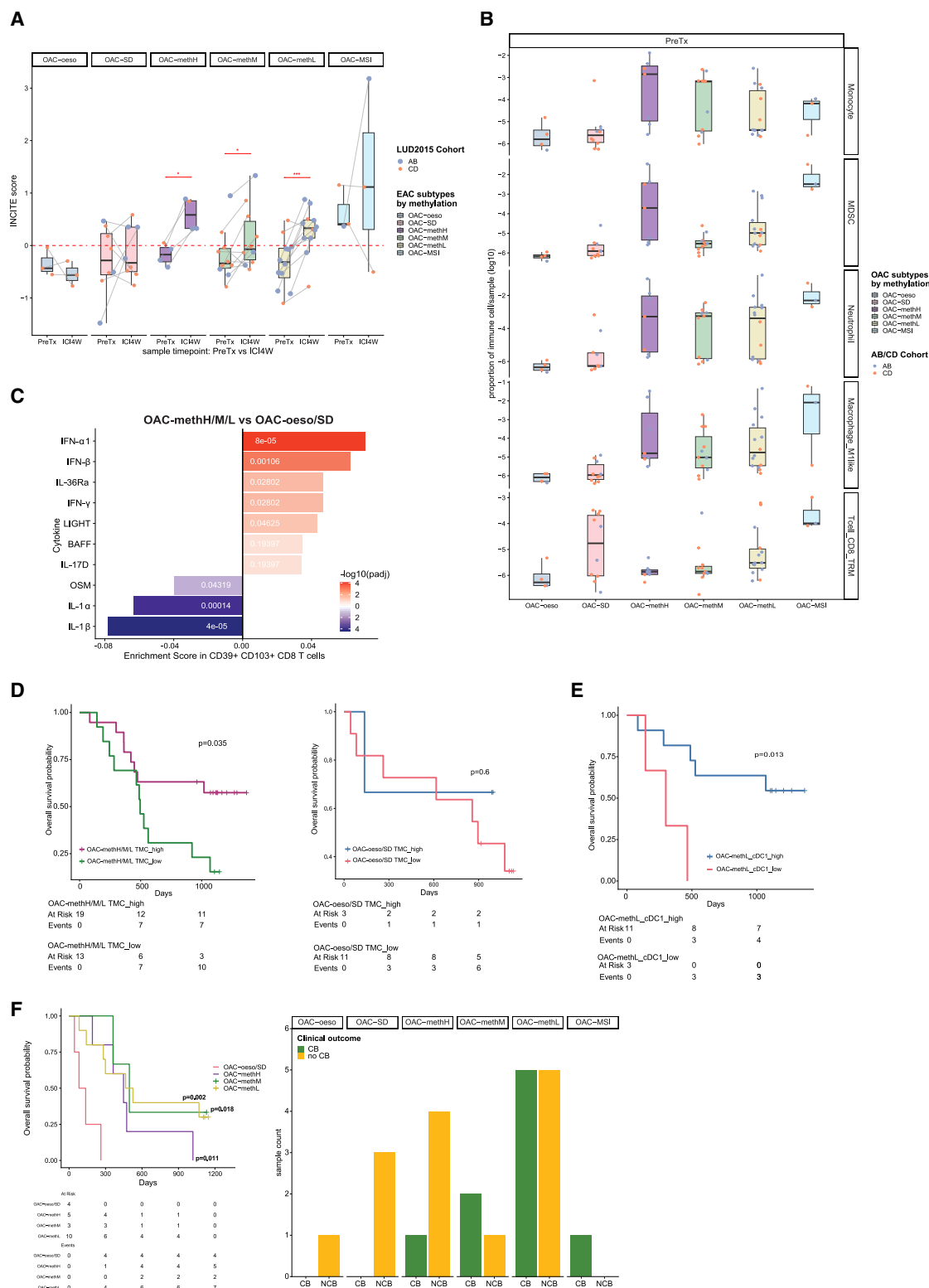


Figure 7. Distinctive tumor DNA methylation subtypes stratify tumor immune microenvironment and clinical response to immunochemotherapy

(A) Boxplot showing INCITE score change per patient grouped by five tumor DNA methylation-derived tumor subtypes (TDM-TS), as well as the MSI subtype, from PreTx to ICI4W (paired *t* test). Patients of LUD2015-inop cohort are denoted with larger blue dots. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

(legend continued on next page)

deconvoluted from the bulk RNA-seq data using our single-cell atlas (Figure 2A). OAC-MethL, -MethM, and -MethH exhibit high fractions of monocyte, neutrophil, myeloid-derived suppressor cells (MDSCs), and M1-like macrophage compared to OAC-oeso and OAC-SD (Figure 7B), while T cell composition is more variable (Figure 7B). CD8⁺ TRM cells are likely to be tumor-reactive and respond to the treatment mediated by higher TMC. Although showing high infiltration in OAC-SD, CD8⁺ TRMs in OAC-oeso/SD express significantly lower type I and II IFN than OAC-methH/M/L (Figure 7C), suggesting reduced CD8⁺ T cell response in the TiME of OAC-oeso/SD.

In line with the observed difference in monocyte infiltration, we observe that TMC is prognostic for patients in OAC-methH, -methM, and methL ($p = 0.035$) but not in OAC-oeso and SD (Figure 7D). For OAC-methH, -methM, and -methL groups, higher infiltration of MDSCs and neutrophils, in addition to TMCs, associates with better survival (Figures S7A and S7B), suggesting the important role of antigen-presenting cells (APC) in ICI response among these three methylation subtypes. Interestingly, for OAC-methL alone, higher infiltration of cDC1 is associated with better survival (Figure 7E), suggesting the distinct type of APC underpinning the response in the OAC-methL subtype. For OAC-oeso and OAC-SD collectively, TMC does not predict survival (Figure 7D, right). All inoperable patients carrying OAC-oeso or OAC-SD tumors have poor prognosis (Figures 7F and S7C), whereas patients carrying OAC-Meth-L/M/H tumor subtypes and with high TMC have gained clinical benefits in response to immunochemo(radio)therapy. Together, the results shown in this study illustrated the power of combining whole genome DNA methylation with tissue transcriptome data and bulk RNA-seq cell deconvolution analysis. It also illustrated that tumor DNA methylation subtypes stratify TiME and can further predict the prognosis of immunotherapy in combination with TMC.

DISCUSSION

The recently reported phase 3 MATTERHORN trial showed a two-year OS rate of 75.7% in patients with OAC treated with neoadjuvant ICI inhibitor plus FLOT (5-FU, leucovorin, oxaliplatin, and docetaxel) chemotherapy (ICI+CTX). This led to the imminent widespread introduction of ICI-based ICI+CTX in late 2025 as standard of care for operable OAC. Survival outcomes were favorable in biomarker-defined subgroups, particularly those with a combined positive score (CPS) > 1, and the to-be-introduced neoadjuvant ICI+CTX treatment will not benefit all patients, underscoring a critical unmet need to identify therapy-resistant patient groups early and understand how treatment strategies can be refined for common benefit.

Here, we report a comprehensive biomarker discovery study in the phase 2 LUD2015-005 trial, which treated 73 patients with OC, including 35 LUD2015-op patients. The 2-year survival rates were 80% (durvalumab + CapOx CTX) and 77.8% (durvalumab + C-FLOT CTX). While the LUD2015-005 trial does not have a standard-of-care treated control arm, propensity score matching of operable OAC patients with patients treated with standard-of-care treatment shows that ICI-based therapy provides a survival advantage in our limited cohort.

In LUD2015-inop, 15/38 patients achieved CB (39.5%, > 1-year PFS¹⁹), comparable with the phase 3 ICI+CTX trials of OSCC, Checkmate-648 (23% PFS >1 year⁵¹), and OAC, Checkmate-649 (25% PFS >1 year¹⁸), which culminated in the regulatory approval of ICI-based combination therapy as a first-line treatment for inoperable OC. In both LUD2015-op and LUD2015-inop cohorts, we showed that immune priming is safe, and our data suggest that this priming plays a role in inducing immune infiltration in immune-excluded tumors, potentially broadening the proportion of patients achieving durable responses with ICI-based combination therapies. Consistent with this, comparable mPR rates to ours were also reported in the PANDA trial, in which 20 patients were treated with a priming cycle of ICI followed by combination with docetaxel, oxaliplatin, and capecitabine chemotherapy.¹⁴ All these suggest that most patients can benefit from ICI-based combination therapy and raise the intriguing possibility that immune priming, as opposed to concurrent therapy, might contribute to anti-tumor efficacy. This is consistent with the observed beneficial effects of neo-adjuvant ICI treatment in melanoma and lung cancer.^{52,53} Future studies are needed to address whether short ICI priming before the ICI-based combination therapy can enhance treatment efficacy, especially in operable OAC.

To identify the molecular basis of heterogeneous patient response to ICI-based therapies, we took advantage of the paired longitudinal tissue sampling from 35 inoperable and 30 operable OC tumors and adjacent normal tissues in the LUD2015-005 trial. The generation of an scRNA-seq tissue atlas and deconvolution of bulk RNA-seq data identified TMC as the feature that best predicts OS in ICI+CTX-treated LUD2015-inop patients.¹⁹ Satisfyingly, using a similar approach, TMC was also identified as the best predictor for survival in operable OC patients receiving durvalumab with chemotherapy or chemoradiotherapy in this study. Further, the generation of a pan-stage OC scRNA-seq atlas identified CFISH as the minimal gene set representative of TMC, establishing both TMC and CFISH as biomarkers to stratify OC patients for ICI-based combination therapy. Direct comparison of CFISH and TMC showed broadly

(B) Boxplot showing the proportions at PreTx of monocyte/sample, MDSC/sample, neutrophil/sample, M1 macrophages, and CD8⁺ TRM T cells (in log10) per patient, grouped by 5 TDM-TS, as well as the MSI subtype.

(C) Bar chart showing the 10 most enriched cytokine signaling pathways by immune response enrichment analysis on the single CD8⁺ TRM cells (CD39⁺, CD103⁺) between OAC-methH/M/L vs. OAC-oeso/SD. Bar length represents the enrichment score, and color reflects the adjusted p value, which is also labeled.

(D) Kaplan-Meier curve comparing overall survival of patients with high TMC and low TMC in OAC-methH/M/L (left), as well as in OAC-oeso/SD (right).

(E) Kaplan-Meier curve comparing overall survival of patients with high and low cDC1 in OAC-methL.

(F) Survival rate and clinical benefit rate of LUD2015-inop with OAC-oeso/SD and OAC-methH/M/L: Kaplan-Meier curve comparing overall survival of inoperable patients with OAC-oeso/SD vs. OAC-methH, -methM, and methL, respectively (left); bar chart showing the clinical benefit rate of LUD2015-inop patients of five TDM-TS and MSI subtypes (right).

similar but modest discriminatory performance for OS by Harrell's C-index and time-dependent AUC analyses (Figure S3D).

CFISH genes are not only expressed in tumor monocytes but also in other myeloid cell types. For example, HLA-DRA is expressed in DCs, whereas CXCL8, FCN1, IFI30, and SLC11A1 are expressed in inflammatory M1-like macrophages^{54–56} and DCs⁵⁶ (Figure S3K). CXCL8 is a neutrophil chemoattractant, FCN1 is a pattern-recognition receptor in innate immunity, HLA-DRA is a major histocompatibility complex (MHC) class II molecule, and IFI30⁵⁷ and SLC11A1⁵⁸ are functionally related to MHC class II antigen presentation. Neutrophils, like macrophages, despite being originally viewed as tumor-promoting, emerge as important cytotoxic T cell supporters in the context of ICI, especially if interferon-stimulated.^{59–61} SLC11A1 expression is associated with favorable ICI response in glioma.⁶² HLA-DRA expression is associated with ICI response in different settings.^{25,63,64} Notably, CXCL8, IFI30, and SLC11A1 expression have been linked with poor outcome from chemotherapy treatment.^{55,62,65,66} Consistent with this, we also observed high CFISH expression links to worse OS in CTX-only-treated patients in the ICGC OC cohort (Figure S3F). The inverse predictive value of CFISH in chemotherapy-treated OC highlights the specificity of this biomarker to ICI response. Furthermore, the finding that the expression of CFISH associated with outcome of ICI-treated cohorts of gastric cancer,^{28,29} melanoma,³⁰ and NSCLC³¹ cohorts demonstrates the potential of CFISH as a powerful predictive biomarker for ICI+CTX-treated cancers beyond OC.

T cells with tumor-reactive TCRs are pre-requisites for a strong anti-tumor immune response.^{25,67} These cells are characterized by CD39/CD103 expression, and their presence correlates with favorable response to ICI-based therapies in a range of studies.^{23,26} But there is a growing appreciation for the importance of myeloid-T cell interactions for a successful cytotoxic anti-tumor response³⁹: DCs present antigens and provide co-stimulation.⁶⁸ Inflammatory macrophages, especially interferon-stimulated and CXCL9-expressing macrophages^{38,39,42,69} and perhaps also neutrophils,^{59–61} support T cell recruitment and function through cytokine secretion while also possibly participating in cancer cell phagocytosis.^{70,71}

Utilizing our pan-stage OC scRNA-seq atlas, we uncover that monocytes are likely to differentiate into LAMP3-high DCs and M1-like macrophages, both of which are specifically linked to favorable ICI response in a range of cancers.^{40,41} Immune response enrichment analysis indicates that this differentiation process is accompanied by IL-15 and IFN signaling, cytokine signaling pathways that are also enriched in responding patients at ICI-4W. The source of DCs in tumors is an area of active research, but LAMP3-high DCs have been suggested to derive from monocytes in culture^{72–75} and bioinformatically,⁷⁶ possibly representing highly activated cDC2-like DCs.⁷⁷ Due to its T cell supportive function, IL-15 agonists are being investigated as immune-stimulatory agents and are showing promising results in clinical trials.⁷⁸ Our data further illustrate a role of IL-15 in shaping the myeloid-related TIME, suggesting a positive feedback loop and providing a link between PreTx monocyte abundance and T cell function. These findings suggest that IL-15 agonists may be able to rescue non-responders to ICI in this

setting, by supporting T cell function even in the absence of monocyte-driven myeloid remodeling.

While total T cell numbers are not related to ICI+CTX/CRTX response or survival in our study, as also reported by others,^{13,15} T cells with a tumor-response phenotype become enriched in patients with favorable outcome. Notably, we observe an increased expression of tumor response markers upon ICI in patients with a LAMP3hi DC and M1-like macrophage-high TIME at ICI-4W, consistent with the notion that myeloid compartment remodeling upon ICI may support tumor-responsive T cells.

Paired tumor biopsy collection from the same patient at PreTx and ICI-4W enabled us to assess every individual's TIME dynamics based on their ability to respond to ICI-only treatment during this window. This is achieved by cell deconvolution of bulk RNA-seq and the measurement of INCITE signature gene expression and associated score. We observe a striking heterogeneity both in PreTx tumor TIME composition and transcriptional response to ICI-only. As reported previously, we detected INCITE gene expression in all LUD2015-inop tumors but with varied INCITE score. The observed high INCITE score might be caused by ICI's ability to overcome highly immune-suppressed and T/NK-cell-excluded TIME in advanced OC tumors.

Within operable OC, some patients' tumors feature a TIME similar to that observed in advanced tumors with sparse CD8⁺ TRM, DC, and NK cell numbers, which underlies INCITE gene expression.¹⁹ Other tumors are abundant in lymphocytes and cDCs at PreTx and show little infiltration with CD8⁺ TRM cells upon ICI-4W. These two operable OC patient groups show distinct transcriptomic changes upon ICI-only treatment: patients exhibiting low immune infiltration before treatment showed INCITE upregulation upon ICI-4W (high INCITE score), as observed in advanced OC, while this gene expression change was absent in patients with higher PreTx immune cell abundance (low INCITE score). The variable state of immunosuppression in operable OC patients generates a varied universal T/NK influx upon ICI. Nonetheless, the proportion of patients benefitting from ICI-based combination therapy was similar in both groups with high or low INCITE scores, consistent with our previous finding that INCITE score alone is not sufficient to stratify OS to ICI-based therapy.

The molecular features of the tumor are closely associated with TIME, both of which are implicated in the efficacy of ICI-based combination therapy. Evidence from the cancer genome atlas project indicates that more tumors share common methylation features compared to somatic genetic alterations.¹² In particular, early methylation array-based studies identified a CpG island methylator phenotype (CIMP) subtype and an Epstein-Barr virus (EBV)-associated hypermethylation type in human gastric cancers.^{20,46,48} However, methylation arrays only cover between 1.5% and 3% of all epigenetically modified CpG positions in the genome, with a strong bias toward CpG islands and their surrounding regions. In addition, most tissue-specifically methylated positions are outside CpG island.²⁰ Therefore, using our recently developed pan-genomic DNA methylation sequencing technology, TAPS, to profile 52 OAC samples derived from 23 inoperable and 29 operable OAC tumor biopsies, combined with the normal and Barrett's esophagus tissues, enhanced our ability to detect tissue- and cell-type-specific DNA methylation patterns. As both

bulk RNA-seq and tissue/tumor TAPS sequencing were derived from the same tumor biopsy in this study, detailed analysis of combined RNA-seq and TAPS data enable us to stratify TiME in association with the tumor epigenetic context. Stratification by TAPS revealed five epigenetically defined subtypes (OAC-MethL, OAC-MethM, OAC-MethH, OAC-oeso, and OAC-SD) within which the predictive value of TMC differed substantially. OAC-MethL/M/H subtypes are more epigenetically divergent and reflect a tumor microenvironment in which monocyte-associated immune dynamics are more tightly linked to therapeutic response. In OAC-oeso and OAC-SD subtypes, whose methylation patterns most closely resemble those of the corresponding normal control tissue (gastric or esophageal), TMC failed to predict OS. One possible explanation is that these more “normal-like” tumors represent an immune-excluded phenotype, in which immune cells are present in the peritumoral stroma but fail to effectively infiltrate or engage the tumor parenchyma. In this context, TMC may not as accurately reflect anti-tumor immune activity, thereby diminishing its predictive value. Additionally, it is also important to note that all inoperable patients carrying OAC-oeso or OAC-SD tumors have poor prognosis, while operable patients with tumors of these two subtypes have varied Mandard score. Future studies with longer follow-up period of operable OAC is needed to determine whether OAC-oeso or OAC-SD groups will also result in poor OS. Together, the findings reported in this study highlight the importance of integrating tumor epigenetic context with immune microenvironmental features to refine predictive biomarkers and improve patient stratification for ICI+CTX.

Limitations of the study

This study reports a comprehensive molecular profiling of the tissue/tumor samples from 73 OC patients (38 inoperable and 35 operable) in LUD2015-005 trial treated with durvalumab either alone or in combination with low or high dose of tremelimumab for 4 weeks, followed by combination therapy consisting of durvalumab plus standard-of-care therapy. The limitations of this study fall into four areas: (1) OAC bias, (2) durvalumab as the main immune priming agent, (3) multiple standard-of-care treatment regimes, and (4) limited sample materials. First, due to the geographical distribution of the cancer types, OSCC is under-represented in this study as most cancers are adenocarcinomas, reflective of the incidence in Europe. In total, only 7/73 patients had OSCC (3 OSCC/38 LUD2015-inop and 4 OSCC/35 LUD2015-op). Second, durvalumab, either alone or in combination with low or high dose of tremelimumab, was used in the immune priming phase to treat inoperable OAC. Tremelimumab in combination with durvalumab failed to show significant impact on patient OS, partly due to the small cohort size for each of the treatment groups. Third, since the start of this trial in 2015, the FLOT regime became standard-of-care chemotherapy for operable OC, requiring a change in regime from CapOX to FLOT during recruitment of the operable OC cohort. As a result, 11 patients received durvalumab+CapOx, and 9 patients received durvalumab+FLOT. We also have CROSS as standard-of-care chemoradiotherapy regime (Figure 1). Due to the limited cohort size, it is impossible to compare the impact of the two chemotherapy regimens in

combination with durvalumab. Fourth, tissue/tumor biopsies collected are limited in size and number as we can only collect 2-mm pinch biopsies at baseline from all OC patients enrolled in the trial. Due to sample limitations, our qPCR validation of CFISH expression was limited to 12 patients, and we have limited capability to carry out immunohistochemistry and spatial biology analysis. Our work on the predictive value of DNA methylation tumor subtypes is exploratory, and while out of the scope of the present manuscript, the use of CFISH and DNA methylation tumor subtypes as predictive clinical biomarkers should be prospectively validated in future work.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xin Lu (xin.lu@ludwig.ox.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw WGS, TAPS, bulk RNA-seq, and scRNA-seq FASTQ files generated from LUD2015-005 patient samples will be deposited at the European Genome-phenome Archive (EGA) and made publicly available (EGA: EGAS00001008543). LUD2015-005 clinical outcome data can be found in Tables S1, S2, and S3. All processed data and reproducible code has been deposited at Zenodo and is publicly available (Zenodo: <https://doi.org/10.5281/zenodo.22067581>). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

For the purpose of Open Access, the author has applied a CC-BY public copyright license to any Author Accepted Manuscript (AAM) version arising from this submission.

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Some terminology has been revised from British to American spelling by editorial requirement; abbreviations based on the prior spelling have been retained for consistency.

AUTHOR CONTRIBUTIONS

H.S.F., T.M.C., R.P.O., M.R.M., and X.L. designed and oversaw data curation and analysis of LUD2015-005 trial samples. M.R.M. is the clinical lead and oversaw the LUD2015-005 trial protocols. G.G., M.L., and S.W. performed clinical statistical analysis. S.M., T.U., I.K., R.P., L.G., K.J.McC., C.P., M.L., D.R.McG., and K.M.B. were involved in implementing the LUD2015-005 trial protocol and acquiring related clinical data and samples. H.S.F., S.A.J., J.A.C., P.X., and T.M.C. performed single-cell RNA-seq and bulk RNA-seq. H.S.F. carried out RNA-seq and scRNA-seq bioinformatic analysis including cell deconvolution analysis and identification of CFISH as tumor monocyte-related five-gene signature. H.S.F. also replicated the initial findings reported in Carroll et al., *Cancer Cell*, 2023. S.A.J. and B.-A.J. performed q-PCR to validate CFISH expression in a selection of 12 tumor samples.

P.X. bi-dissected tumor samples and extracted DNA and RNA from the same biopsy for the WGS/TAPs and bulk RNA-seq. With the help of M.I., P.S.-Z., S.K., E.S., and P.X. acquired DNA methylation data using TAPS assay and set up the initial DNA methylation analysis of TAPS-seq data with assistance from E.W. Z.C. performed TAPS data analysis and integrated it with bulk RNA-seq, with input from P.X., to identify five DNA methylation subtypes. C.-X.S. and S.K. supervised TAPS assay. B.S.-B. and R.P.O. oversaw the computational analysis of WGS, TAPS, single-cell RNA-seq, and cell deconvolution analysis. B.A. and D.P. curated all sequencing data and organized appropriate storage in the cluster ready for analysis and data repository upload. Currently, Z.C., E.W., D.P., B.S.-B., and R.P.O. have full access to all sequencing data.

H.S.F. prepared the first draft of the manuscript with the help of R.P.O., S.A.J., P.X., and Z.C. X.L., B.S.-B., and M.R.M. reviewed and edited the manuscript. X.L. oversaw the project and the manuscript and acquired funding from LICR. X.L. and M.R.M. acquired funding from CRUK and NIHR and support from AstraZeneca UK Limited. Dr. Kate Dunning provided editorial assistance. All authors read and approved the final article to be submitted to *Med* and take responsibility for its content.

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STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODELS AND STUDY PARTICIPANT DETAILS**
 - LUD2015-005 trial design
 - LUD2015-op
- **METHOD DETAILS**
 - Assessment of patient outcomes in LUD2015-op
 - Research study procedures and sample collection
 - Single-cell RNA sequencing
 - Bulk tissue RNA sequencing
 - Whole genome sequencing
 - Whole genome DNA methylation sequencing
 - Reverse transcription and quantitative PCR (qPCR)
 - Bioinformatics
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **ADDITIONAL RESOURCES**

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Durvalumab	AstraZeneca UK, Ltd	N/A
Tremelimumab	AstraZeneca UK, Ltd	N/A
Biological samples		
Biopsies of tumor and normal upper gastrointestinal tract tissues from esophageal cancer patients	LUD2015-005 clinical trial	NCT02735239, EudraCT 2015-005298-19
Chemicals, peptides, and recombinant proteins		
Heat-inactivated human serum	Sigma-Aldrich	Cat#H3667
Fetal Bovine Serum	Sigma-Aldrich	F9665
DMEM/F-12	Gibco	Cat#11330032
PBS	Gibco	Cat#11590476
Bovine Serum Albumin Fraction V	Apollo Scientific	Cat#BIA3981
Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1)	Invitrogen	Cat#AM9720
TURBO DNA-free Kit	ThermoFisher	Cat#AM1907
Red Blood Cell Lysis Solution (10×)	Miltenyi	Cat#130-094-183
Animal Free Collagenase/Dispase Blend II	Sigma-Aldrich	Cat#SCR140
DNAse I	Sigma-Aldrich	Cat#D5025
Collagenase D	Sigma-Aldrich	Cat#11088866001
Liberase DL	Sigma-Aldrich	Cat#5466202001
Oxaliplatin	Eloxatin	N/A
Capecitabine	Xeloda	N/A
Nuclease-free water	Ambion	AM9937
qPCR primers	ThermoFisher	N/A
mTet1CD	Produced in-house per Liu et al. ⁴⁹	N/A
Borane-pyridine complex, 95%	Alfa Aesar	L13178.09
HEPES (pH 8.0)	ThermoFisher	J63578-AP
Ammonium iron (II) sulfate hexahydrate	Merck	09719
Alpha-ketoglutarate	Merck	K3752
Ascorbic acid	Merck	95210
Dithiothreitol (DTT)	Merck	D9779
NaCl	ThermoFisher	AM9759
ATP	Fisher Scientific	10326943
KAPA Hyper Prep Kit PCR-free	Roche	KK8505
KAPA HiFi HotStart Uracil+ Kit	Roche	KK2802
KAPA Unique Dual-Indexed Adapters	Roche	KK8726
Proteinase K	ThermoFisher	EO0491
Sodium acetate	ThermoFisher	J63669.AE
Tris-HCl	ThermoFisher	15567027
Complete Protease Inhibitor Cocktail	Roche	4693116001
Triton X-100	ThermoFisher	A16046.AE
ANTI-FLAG M2 Affinity Gel	Millipore	A2220
Pierce 3X DYKDDDDK Peptide	ThermoFisher	A36805
ExpiFectamine 293 Transfection Kit	Gibco	A14525
Expi293 Expression Medium	Gibco	A1435101

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Opti-MEM Reduced Serum Medium	Gibco	31985070
Critical commercial assays		
Chromium Single Cell Immune Profiling Assay (v2)	10x Genomics	https://www.10xgenomics.com/products/single-cell-immune-profiling
mirVana miRNA Isolation Kit	ThermoFisher	Cat#AM1560
Dead Cell Removal Kit	Miltenyi	Cat#130-090-101
TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Kit	Illumina	Cat#20020596
GeneJET Genomic DNA Purification Kit	ThermoFisher	Cat#K0722
Qubit RNA BR Assay	ThermoFisher	Cat#Q10211
Qubit dsDNA HS Assay	ThermoFisher	Cat#Q32851
Tapestation High Sensitivity DNA Kit	Agilent	5067-4626
Tapestation High Sensitivity RNA Kit	Agilent	5067-5579
HiScript IV RT SuperMix for qPCR (+gDNA wiper)	Vazyme	R423
SYBR qPCR Master Mix	Vazyme	Q713
Deposited data		
LUD2015-inop demographics and clinical outcomes (cut-off date 16 October 2020)	This paper	Tables S1, S2, and S3
LUD2015-inop bulk RNA sequencing	Carroll et al. ¹⁹	EGA :EGAS00001006468
LUD2015-inop single-cell RNA sequencing	Carroll et al. ¹⁹	EGA :EGAS00001006469
LUD2015-inop whole genome sequencing	Carroll et al. ¹⁹	EGA :EGAS00001006470
International Cancer Genome Consortium: ESAD-UK cohort RNA sequencing counts, mutational burden	Frankell et al. ³²	EGA :EGAD00001004423; https://dcc.icgc.org
Bulk RNA sequencing data for ICI-treated gastric cancer (Samsung cohort)	Kim et al. ²⁹	ENA: PRJEB25780
Bulk RNA sequencing data for ICI-treated gastric cancer (Yonsei and St. Mary's cohorts)	Cheong et al. ²⁸	EGA: EGAD00001008091
Control dataset of patients with upper gastrointestinal cancers treated in the local NHS Trust during the treatment period of the LUD2015-005 study	Oxford University NHS Foundation Trust	NA
Processed RNAseq counts from ICI-treated melanoma	Gide et al. ³⁰	ENA: PRJEB23709
Processed RNAseq counts from ICI-treated NSCLC	Cho et al., ³¹	GEO: GSE135222
LUD2015-op demographics and clinical outcomes (cut-off date 22 November 2022)	This paper	Tables S1 and S3 , this paper.
LUD2015-op bulk RNA sequencing	This paper	EGA: EGAS00001008543
LUD2015-op single-cell RNA sequencing	This paper	EGA: EGAS00001008543
LUD2015-op whole genome sequencing	This paper	EGA: EGAS00001008543
Whole genome methylation data for LUD2015-inop (23 patients), and LUD2015-op (29 patients), Barretts2016 (9 samples from 2 patients)	This paper	EGA: EGAS00001008543
Software and algorithms		
DESeq2 v1.38.3	Love et al., ⁷⁹	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Cell Ranger v7.1.0	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/3.1/
BayesPrism v2.0	Chu et al., ²⁷	https://github.com/Danko-Lab/TED/
Seurat v4.3.0	Stuart et al., ⁸⁰	https://satijalab.org/seurat/index.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
sctransform v0.3.5	Hafemeister and Satija, ⁸¹	https://cran.r-project.org/package=sctransform
FastMNN method	Haghverdi et al., ⁸²	https://bioconductor.org/packages/release/bioc/html/batchelor.html
survivalAnalysis v0.3.0	CRAN (Wiesweg)	https://cran.r-project.org/package=survivalAnalysis
survival 3.8.3	Therneau and Grambsch, 2000 ⁸³	https://CRAN.R-project.org/package=survival
compareC 1.3.3	Kang et al., 2015 ⁸⁴	https://CRAN.R-project.org/package=compareC
timeROC 0.4.1	Blanche et al., 2013 ⁸⁵	https://CRAN.R-project.org/package=timeROC
MatchIt v4.5.5	Ho et al., ⁸⁶	https://github.com/kosukeimai/MatchIt
R v4.2.2	R Core Team	https://www.r-project.org
R v4.3.2	R Core Team	https://www.r-project.org
pheatmap v1.0.12	CRAN (Kolde)	https://cran.r-project.org/package=pheatmap
ggplot2 v3.4.3	Wickham, 2016 ⁸⁷	https://ggplot2.tidyverse.org/
ComplexHeatmap 2.18.0	Gu, 2016 ⁸⁸ ; Gu, 2022 ⁸⁹	https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html
uwot 0.2.2	Melville, 2024 ⁹⁰	https://CRAN.R-project.org/package=uwot
Nextflow pipelines for WGS and bulk RNA sequencing pre-processing and alignment, including references for software dependencies	This paper	Zenodo: https://doi.org/10.5281/zenodo.22067581 (archive of bitbucket.org/licroxford/fuchs_et_al._2026)
Pipelines for mutation and copy number calls	This paper	Zenodo: https://doi.org/10.5281/zenodo.22067581 (archive of bitbucket.org/licroxford/fuchs_et_al._2026)
Reproducible code for downstream analysis and figure generation	This paper	Zenodo: https://doi.org/10.5281/zenodo.22067581 (archive of bitbucket.org/licroxford/fuchs_et_al._2026)
Pipelines for TAPS sequencing pre-processing, alignment and methylation calling	This paper	Zenodo: https://doi.org/10.5281/zenodo.22067581 (archive of bitbucket.org/licroxford/fuchs_et_al._2026)
Reproducible code for methylation profiling and analysis, as well as figure generation	This paper	Zenodo: https://doi.org/10.5281/zenodo.22067581 (archive of bitbucket.org/licroxford/fuchs_et_al._2026)
GENCODE v36 GTF	Api.gdc.cancer.gov	https://api.gdc.cancer.gov/data/be002a2c-3b27-43f3-9e0f-fd47db92a6b5
velociraptor (v1.8)	CRAN (Rue-Albrecht, 2020) ⁹¹	https://bioconductor.org/packages/release/bioc/html/velociraptor.html
Velocity.R (v0.6)	La Manno et al., 2018 ⁹²	https://github.com/velocity-team/velocity.R
Monocle3 (v1.3.4)		https://cole-trapnell-lab.github.io/monocle3/docs/introduction/
Trim Galore! (v0.6.7)	Krueger et al., 2021 ⁹³	https://github.com/FelixKrueger/TrimGalore
BWA-MEM2 (v2.2.1)	Li et al., 2013 ⁹⁴	https://arxiv.org/abs/1303.3997
rastair (v0.8.1)		https://docs.rastair.com/index.html
gatk (v4.4.0.0)	Van der Auwera & O'Connor (2020) ⁹⁵	https://gatk.broadinstitute.org/hc/en-us/articles/360036194592-Getting-started-with-GATK4
ichorCNA (v0.2.0)	Adalsteinsson et al., ⁹⁶	https://github.com/broadinstitute/ichorCNA
HATCHet (v2.1.0)	Myers et al., 2024 ⁹⁷	https://github.com/raphael-group/hatchet
Other		
Flowmi Cell Strainers, 70uM for 1000uL pipette tips	Sigma-Aldrich	Cat#BAH136800070
CellTrics 100µM filter	Sysmex	Cat#04-004-2328
Fisherbrand™ RNase-Free Disposable Pellet Pestles	Fisher Scientific	Cat#13236679
AMPure XP	Beckman Coulter	Cat#A63880
SPRIselect	Beckman Coulter	Cat#B23317

EXPERIMENTAL MODELS AND STUDY PARTICIPANT DETAILS

LUD2015-005 trial design

The LUD2015-005 clinical trial is a phase 1/2 study of durvalumab as first-line therapy for patients with esophageal or gastro-oesophageal cancer (NCT02735239). Adult patients with a histologically confirmed diagnosis of locally advanced or metastatic esophageal or gastro-oesophageal cancer were eligible for enrollment into the inoperable arms of the LUD2015-005 trial (LUD2015-inop). Patients with operable esophageal or gastro-oesophageal cancer were eligible for enrollment into the operable arms (LUD2015-op), determined by multidisciplinary team meeting. Patients with previous systemic anti-cancer therapy for this same advanced disease were excluded from this study, as were patients who had been treated with ICI in any previous setting. Inclusion criteria included an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and an anticipated lifespan of greater than 4 months. Details of the LUD2015-inop cohorts were previously published.²⁵ Written informed consent was obtained for all study participants. Participants information on sex, age, and race was self-reported. Information on gender and socioeconomic status was not collected. South Central - Hampshire A Research Ethics Committee approved the study (16/SC/0015).

LUD2015-op

Tolerability and safety of 750 mg of intravenous durvalumab was established for the LUD2015-inop cohorts. Thereafter, eligible patients were enrolled into ICI + CTX or ICI + CRTX treatment arms for operable patients based on clinical considerations. CTX vs. CRTX treatment decisions were made in the multidisciplinary team meeting setting. Patients ($n = 35$) received 750 mg of intravenous durvalumab administered biweekly (Q2W).

After 4 weeks of treatment with this ICI agent alone (ICI-only), ICI was combined with CTX or CRTX: patients in cohort C-CapOx received oxaliplatin every three weeks and daily capecitabine for 6 weeks. Patients in cohort C-FLOT received 5-Fluorouracil, Leucovorin, Oxaliplatin and docetaxel every 2 weeks for two months.²¹ C-FLOT patients could also receive FLOT post-operatively. Cohort D received paclitaxel and carboplatin once weekly along with daily radiotherapy for five weeks according to the CROSS regime.²² Initially, cohort D received CRTX alone after ICI, with a further ICI dose after CRTX was completed, and up to 12 cycles post-surgery (cohort D, $n = 12$). After safety review by the Scientific Review Committee, additional ICI was approved to be given in combination with CRTX, and up to 12 cycles post-surgery (cohort D2, $n = 3$). In the absence of a reason to discontinue treatment earlier, durvalumab infusions continued until the end of the final CRTX cycle and for up to 12 cycles post-surgery. The demographic details of the 35 operable patients who received treatment on the LUD2015-005 trial can be found in Table S3. A CONSORT flow diagram for LUD2015-op outlining recruitment and population size per cohort for various stages of the trial can be found in Figure S1A.

METHOD DETAILS

Assessment of patient outcomes in LUD2015-op

The primary outcomes of this phase 2 study involved safety: the number of subjects reporting adverse events (up to one year after first dose of study medication), and number of subjects experiencing a dose-limiting toxicity (up to 10 weeks after first dose of study medication). Secondary outcomes were overall survival at the end of study, progression-free survival and tumor response. Response was assessed using Immune-related Response Evaluation Criteria in Solid Tumors (irRECIST) criteria⁹⁸ and PERCIST criteria from ¹⁸FDG PET/CT scans conducted during the screening period and during treatment. irRECIST outcomes were classified as complete responses (irCR), partial responses (irPR), progressive disease (irPD), or stable disease (irSD). PERCIST responses were classified as complete metabolic response (CMR), partial metabolic response (mPR) and progressive disease (PD). Overall survival (OS) and progression-free survival (PFS) were calculated including data from the post-study follow-up period. Overall survival is defined as the time from start of treatment until death or censored at the latest date the patient was seen or assessed and known to be alive. PFS is defined as time from start of treatment until disease progression as defined by irRECIST criteria (i.e., confirmed by two consecutive scans or unequivocal clinical progression) or death. Those progression-free and alive were censored at the latest date the patient was seen or assessed and known to be alive. Major pathological response was also detected from core biopsies taken from resection specimens at the day of surgery using the Mandard scoring system.⁹⁹ A Mandard score of 1 or 2 was defined as major pathological response. R⁰ resection was defined as absence of residual tumor within 1mm of the surgical margin on microscopy. The statistical analysis plan was defined and approved prior to trial commencement and completed after the trial was concluded by a senior trial statistician, with quality control performed by a trial statistician.

Research study procedures and sample collection

Endoscopic biopsies were collected prior to the onset of treatment, during the fourth week of treatment with ICI alone (ICI-4W, prior to the onset of chemotherapy), and at the end of the study treatment protocol (PostTx). At each endoscopy, up to five 2 mm biopsy pairs were taken from the site of the tumor, while 1–2 pairs of normal control tissue were also taken from the gastric cardia (at least 2 cm distal to the gastro-oesophageal junction (GEJ) or distal extent of lesion if crossing the 1000 GEJ), duodenum and normal esophagus (at least 2 cm proximal to the GEJ or proximal extent of lesion, whichever is more proximal). Each biopsy pair was split into two aliquots, one of which was immediately snap-frozen using dry ice or liquid nitrogen, while the other was slow frozen with 1 mL of fetal

bovine serum (FBS) or heat-inactivated human serum with 10% DMSO and placed at -80°C in a controlled-rate freezing container. For LUD2015-op patients core biopsies were collected from surgical specimens on the day of surgery. Core biopsies from LUD2015-005 surgical specimens and the supplemental biopsies from Barrett's esophagus patients were collected fresh in a 1.5 mL microcentrifuge tube containing DMEM/F-12 with 10% human serum and were kept on ice until processing. Snap and slow-frozen biopsies collected at local enrollment sites were stored at -80°C and were shipped on dry ice to the central site for long term storage at -80°C and subsequent analysis. For downstream nucleic acid extraction, snap frozen tumor samples were retrieved from storage and bi-dissected on dry ice, with each half used for RNA and DNA extractions respectively.

Single-cell RNA sequencing

Tissue dissociation

Prior to dissociation, slow-frozen biopsies were thawed in a 37°C water bath. For both fresh and frozen material, biopsies were washed in PBS, then transferred into either fresh or frozen tissue dissociation medium (Table S6) and dissociated under constant rotation in an incubator at 37°C and 5% CO_2 for 45 min. The dissociated solution was then filtered through a $100\ \mu\text{M}$ CellTrics filter and the resulting filtrate was centrifuged to pellet cells. For fresh material, red blood cell lysis was performed using red blood cell lysis solution and dead cells were depleted using a magnetic bead-based approach. The resultant cell pellets were then resuspended in pre-chilled cell resuspension buffer (0.04% Bovine Serum Albumin in PBS) and filtered through a $70\ \mu\text{M}$ Flowmi tip strainer. Filtered cell suspensions were then counted using an automated cell counter (Bio-Rad, TC20), diluted to a concentration of approximately 1 million total cells/mL in cell resuspension buffer, and kept on ice until encapsulation.

Encapsulation, library preparation, and sequencing

scRNAseq was conducted using a 5' scRNAseq gene expression workflow (Chromium Single Cell Immune Profiling, Solution v1.1 and v.2, 10x Genomics). Encapsulation of cells was performed using the Chromium Controller, and then GEM-RT, cDNA amplification, and construction of final libraries was conducted following manufacturer's instructions. Size profiles of amplified cDNA and final sequencing-ready libraries was verified by on-chip electrophoresis (Agilent 2100 Bioanalyzer system or Agilent TapeStation) using the High Sensitivity DNA Kits, and concentration of final libraries was assessed by Qubit. Libraries were sequenced on an Illumina NovaSeq 6000 or NextSeq 500 or NextSeq 2000 (26 cycles read 1, 8 cycles i7 index, 98 cycles read 2), targeting a minimum of 20,000 reads per cell.

Bulk tissue RNA sequencing

Total RNA was extracted from endoscopic biopsies using the mirVana miRNA Isolation Kit. Briefly, snap-frozen biopsies were transferred directly into pre-chilled lysis/binding buffer in a 1.5 mL microcentrifuge tube, and the tissue was homogenized on ice using a disposable RNase-free pestle. Acid-Phenol:Chloroform RNA extraction was then performed following manufacturer's instructions. Genomic DNA was digested from the eluted RNA using a 30-min incubation with TURBO DNase. RNA concentration 1050 was then calculated using the Qubit RNA BR Assay and the RNA integrity number (RIN) was calculated using the Agilent 2100 Bioanalyzer system (Total RNA Assay). RNA was then plated and stored at -80°C . Prior to library preparation, thawed RNA was purified and concentrated using AMPure XP at a 2.8x ratio. Bead-based rRNA depletion and total RNA sequencing library preparation with dual sample indexing was then conducted using the TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Kit. Libraries were sequenced on the Illumina HiSeq or NextSeq 2000 (75 cycles read 1, 8 cycles i7 index, 8 cycles i5 index, 75 cycles read 2) to a targeted depth of 50 million reads per library.

Whole genome sequencing

Genomic DNA was extracted from endoscopic biopsies using GeneJET Genomic DNA Purification Kit according to manufacturer's protocol. DNA quality and quantity was measured using Nanodrop and Qubit dsDNA HS assay kit. Extracted DNA samples were then sent out for library preparation using a PCR-free protocol and sequencing using the Illumina NovaSeq6000.

Whole genome DNA methylation sequencing

TAPS was performed on all tumor biopsy samples and selected normal controls to obtain whole-genome DNA methylation at single-base resolution. Details of the protocol were previously described.⁴⁹ Briefly, genomic DNA was fragmented using Covaris M220 ultrasonicator, followed by size selection for fragments between 200–400 bp using AMPure XP beads. Adaptor ligation was performed using KAPA HyperPrep PCR-free Kit (KK8505) with methylated and unmethylated spike-in controls, followed by TET oxidation and borane reduction. The resulting libraries were then amplified for 4 cycles using KAPA HiFi HotStart Uracil+ReadyMix Kit (KK2802), and pooled together before sending out for sequencing using the Illumina NovaSeq6000 at 30x target coverage for tumor and 15x for normal tissues.

Reverse transcription and quantitative PCR (qPCR)

Reverse transcription and quantitative PCR (qPCR) for *CXCL8*, *FCN1*, *IFI30*, *SLC11A1* and *HLA-DRA* were performed using Vazyme kits R423 and Q713, respectively. Briefly, 1 μg of total RNA was used for genomic DNA removal followed by first-strand cDNA synthesis. For qPCR, 1/40 of the resulting cDNA was used per reaction. Primers were used at a final concentration of 0.2 μM .

Target	Primer	Sequence
CXCL8	Forward	GAGAGTGATTGAGAGTGGACCAC
	Reverse	CACAACCTCTGCACCCAGTTT
FCN1	Forward	TGGCTCTGTGGACTTCTATCGG
	Reverse	CCTCAAAGTCCACCAGGTCTAC
IFI30	Forward	GTGGGAGTTCAAGTGCCAGCAT
	Reverse	GCAGACAATGGTCAGGAAGGCT
SLC11A1	Forward	CATCCTCACGTTCAACAGCATG
	Reverse	CCACGAAGTAGAGGTTGATGGC
HLA-DRA	Forward	AGCTGTGGACAAAGCCAACCTG
	Reverse	CTCTCAGTTCACAGGGCTGTT
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
	Reverse	ACCACCCTGTTGCTGTAGCAA

qPCR was conducted on a QuantStudio 3 instrument (ThermoFisher Scientific) using thermocycling conditions recommended by the manufacturer. Gene expression was analyzed using the delta threshold cycle (ΔC_t) method, with normalisation to the house-keeping gene *GAPDH*.

Bioinformatics

Repository data access

For CFISH analysis, public bulk RNAseq data for ICI-treated gastric cancer was downloaded from ENA accession PRJEB25780²⁹ and EGA accession EGAD00001008091.²⁸ ICGC EAC raw sequencing and outcome data were downloaded from EGAD00001004423.³² Processed bulk RNAseq counts from ICI-treated melanoma (PRJEB237092³⁰) and NSCLC (GSE135222³¹) were accessed through the TIGER platform.¹⁰⁰

Whole genome sequencing data analyses

Whole genome sequencing data were processed for alignment to the GRCh38 human genome, mutation calling, and copy number analysis in line with current best practices using a set of fully reproducible Nextflow¹⁰¹ pipelines. All pipeline definitions and associated information can be found at <https://doi.org/10.5281/zenodo.22067581> (archive of bitbucket.org/licroxford/fuchs_et_al_2026).

Briefly, mutations were called using a consensus of two of three callers (Strelka2, Mutect2 and Octopus). Following previous practice,¹⁰² tumor mutational burden (TMB) was quantified as the rate of non-synonymous mutations, including single nucleotide variations (SNV) and insertions and deletions (INDEL), in the coding space, reported as mutations per megabase. The size of the coding space used in this study was 35.6 Mb, calculated as the sum of the protein-coding exons from ENSEMBL100 gene annotation. The samples with count of INDEL mutations higher than 20,000 were denoted as MSI subtype.

Large-scale genome copy number aberrations (CNA) were inferred by ichorCNA 0.2.0⁹⁶ and HATCHet 2.1.0.¹⁰³ The purity estimate per tumor sample was calculated based on the CNA profiling of ichorCNA. The large-scale genome instability was profiled through aneuploidy scoring (chromosome arm ploidies), which was calculated through the pipeline developed by Shukla et al.¹⁰⁴ In particular, the aneuploidy score was calculated on the major clone within the selected output from HATCHet that produces the closest purity estimate to ichorCNA.

Single-cell RNA sequencing data analyses

Processing of scRNAseq data was conducted using the Cell Ranger pipeline from 10x Genomics. Briefly, raw BCL files were converted to demultiplexed FASTQ files using cellranger mkfastq. A custom reference sequence was then prepared using the concatenation of the GRCh38 human genome (ENSEMBL100) and supplementary contigs from the Genomic Data Commons alignment reference, namely the decoy contig hs38.d1 and a collection of viral genomes commonly found in human cancer samples. The full GENCODE v34 GTF was used as a gene annotation file, filtering out readthrough transcripts and annotations to PAR regions in chromosome Y as per Cell Ranger recommendations. A custom Cell Ranger reference set was then prepared from these sequence and annotation files using cellranger mkref with default arguments. Finally, the identification of true cells and construction of a UMI counts table for each sample was performed using cellranger count, and these per-sample counts were combined into a single matrix for downstream analysis using cellranger aggr without normalization.

Downstream analysis was conducted using Seurat.⁸⁰ Low-quality cells with less than 200 genes detected or more than 25% mitochondrial reads were removed. For initial clustering of the full dataset, we normalized using SCTransform,⁸¹ performed UMAP dimensionality reduction, and identified clusters with the default Louvain algorithm. Broad cell type partitions were then identified and subset into separate Seurat objects. To characterize smaller cell subsets, the above workflow was repeated on each of these partitioned objects. To minimize batch effect between fresh and frozen samples for these smaller objects, we conducted SCTransform normalization on fresh and frozen samples separately, and then integrated these two datasets using FastMNN,⁸² a mutual nearest neighbor

batch correction method, before proceeding to dimensionality reduction. Marker genes for each identified cluster were calculated using the FindAllMarkers function, and these markers were used to manually assign cell type and cell subtype labels.

Clusters representing low-quality cells, detected based on abnormally high mitochondrial percentage, increased levels of a gene signature representative of dissociation-induced stress,¹⁰⁵ and/or an abnormally low number of features within a given cell type, and clusters representing doublets, detected based on marker expression with an abnormally high number of features as supporting evidence, were identified and removed from the final scRNAseq object.

Trajectory analysis on sub-compartments was performed using monocle 3 (v1.3.4). RNA velocity analysis was performed using both intronic and exonic reads. As intronic reads are discarded by cellranger count, alignment was additionally performed with velocity (v0.17.17), aligning to the same reference genome, to generate loom files containing both spliced and unspliced reads. These were aggregated into a SingleCellExperiment (v1.20.1). The previously generated Seurat object of the sub-compartment of interest was also converted into a SingleCellExperiment. The SingleCellExperiment, newly generated from loom files, was subset for high-quality cells belonging to the sub compartment of interest and spliced and unspliced reads were added as additional assays to the myeloid SingleCellExperiment. RNA velocity analysis was conducted with velocytor (v1.8), an R wrapper for scvelo.

Immune response enrichment analysis was performed using the immune dictionary.³⁶ Cell-cell communication analysis was performed using NicheNetR (v2.2). Figures visualizing the scRNAseq findings were generated using a combination of Seurat and ggplot2.

Bulk RNA sequencing analysis

Raw RNA sequencing data were processed following best practices using a reproducible Nextflow¹⁰¹ pipeline. This workflow was based of STAR¹⁰⁶ alignment to the same reference used for scRNAseq alignment, and generation of final counts matrices using featureCounts.¹⁰⁷ The reproducible pipeline and associated information can be found at <https://doi.org/10.5281/zenodo.22067581> (archive of bitbucket.org/licroxford/fuchs_et al. _2026).

Differential expression was conducted using DESeq2 using a significance threshold of FDR <0.1 to identify differentially expressed genes. Default arguments were used, except for the use of the “local” fit type to model dispersion estimates. Moderated gene-level log-fold changes were calculated within DESeq2 using the “ashr” method.^{79,108}

Log-normalized counts for heatmap visualisation were generated using the varianceStabilizingTransformation (vst) function. To assess changes between timepoints while correcting for patient-specific effects, a design formula of ~patient+timepoint was used.

Deconvolution analysis

All major cell types identified from scRNAseq were used for bulk RNAseq deconvolution with BayesPrism,²⁷ which performed well on a previous benchmarking study using LUD2015-inop samples.¹⁹ For all deconvolution analyses, normalized counts in a linear scale were used for deconvolution, which were obtained for scRNAseq data using the vst function from the SCTransform package and for bulk RNAseq data using the median of ratios method embedded in the estimateSizeFactors function from DESeq2. The combination of these normalization methods has been shown to provide accurate results across a variety of deconvolution approaches.¹⁰⁹

Potentially confounding gene types (mitochondrial, ribosomal, sex-specific, and TCR/BCR variable region genes) were excluded from the input reference matrix prior to each run. An input matrix was generated using the 3000 most variable genes (as calculated by FindVariableFeatures from Seurat) and cell type and subtype labels were additionally supplied from the scRNAseq object, specifying EAC as the malignant cell type for adenocarcinomas, and ESCC for squamous cell cancers, which were separately deconvoluted. Samples from LUD2015-inop and LUD2015-op were also separately deconvoluted.

Normalized and scaled cell type proportion estimate for individual cell types were used to perform Cox regressions against overall survival and estimates for cell types within broad cellular compartments were used to perform hierarchical clustering of samples (using the hclust method with “ward.D” linkage).

Linear mixed effect modeling was conducted to assess the relationship between the abundance of monocytes and other cell types using the lme4 (v.1.36) package. Log10-normalised monocytes and sampling timepoints were defined as fixed effects and patient ID as random effects with the following design formula: lmer(Cell_Abundance ~ timepoint * Monocyte + (1 | patient)). Significance testing was performed using the Satterthwaite degrees of freedom estimation.

Generation of CFISH gene set

Monocyte markers were identified from the scRNAseq atlas using the FindMarkers function, comparing cells annotated as monocytes to all other cells. Best subset regression was performed on the 30 most enriched markers using the leaps (v.3.2) package using default settings without nesting or predetermined weighting. An optimal subset of 10 markers was determined using the Bayesian information criterion (BIC) and Mallow’s CP value. A multivariate linear model without nesting was constructed using the qr method and log-transformed monocyte abundance, as a proportion of total cells as determined by deconvolution, as the response variable. All five genes with a positive coefficient (CXCL8, FCN1, IFI30, SLC11A1, HLA-DRA) were used to define the CFISH gene set. CFISH scores were calculated from summed vst-normalised expression of the five genes.

Survival analysis

The link between variables and survival outcomes were assessed using the survivalAnalysis package in R. analyze_survival was used to conduct univariate Cox regressions and to generate Kaplan-Meier plots, while analyze_multivariate was used for multivariate Cox regressions and forest plots. Unless otherwise noted, continuous variables were scaled and centered prior to regression. The comparisons of discriminatory performance of TMC and CFISH for OS were conducted using Harrell’s C-index and time-dependent AUC at 1, 2, 3 years. C-index was assessed using the survival package in R. The 95% CI of C-index was estimated using bootstrap

resampling. The p -value of C-index was measured using compareC package. Time-dependent AUC analysis, as well as the p -value calculation, was conducted using timeROC package.

Propensity score matching was performed with 403 OAC patients treated using CapOx, FLOT, or CROSS treatment protocols, but did not receive ICI, in the local NHS Trust (Oxford University Hospital NHS Trust) during the same period. This data was extracted from an Oxford-based database, the Clinical Outcomes Database Application (CODA). For propensity matching analysis, patient treatment, demographic, and clinical outcome data were extracted and only patients with stage II, III or IV esophageal adenocarcinomas treated with neoadjuvant chemotherapy or chemoradiotherapy were included. Patient age, Charlson comorbidity index, N stage, and neoadjuvant treatment approach were identified as significantly different between LUD2015-op and CODA, and were hence used for propensity scoring. Nearest neighbor 1:1 matching between LUD2015-op and the CODA subset was performed using the MatchIt package.⁸⁶

TAPS sequencing data preprocessing

Raw sequencing reads were trimmed from adapter sequences and low-quality bases using Trim Galore! (<https://zenodo.org/record/5127899>) (v0.6.5) and assessed for quality with FastQC (S. Andrews, FastQC (Babraham Bioinformatics, Babraham Institute, 2010).) (v0.11.9). Reads were aligned to the reference genome using BWA-MEM2 (v2.2.1)¹¹⁰ and duplicates were marked with Picard (Genomics in the cloud: using Docker, GATK, and WDL in Terra (O'Reilly Media, 2020).) (gatk, v4.4.0.0). BAM files were indexed and merged using samtools (v1.16.1).¹¹¹ TAPS conversion efficiency and methylation bias were estimated by analyzing sequencing spike-ins. Methylation levels were called using rastair (v0.8.1).¹¹²

In addition to the LUD2015-005 trial samples that were TAPS sequenced (23 tumor samples of LUD2015-inop cohort, 29 tumor samples of LUD2015-op cohort, 6 esophageal samples, 2 gastric samples and 3 duodenum samples), we also included whole-genome bisulfite (WGBS) sequenced methylation dataset of the samples donated from two patients to The Oxford Radcliffe Biobank (ORB), denoted in the paper as patient “2011” and “0966” from Cohort “Barretts2016”. The samples of Barretts2016 include 2 Barrett’s esophageal samples, 2 esophageal samples, 2 gastric samples, 2 duodenum samples and 1 high-grade dysplasia sample. The raw sequencing reads were processed following the similar pipeline as the above.

Differentially methylated regions (DMRs) identification

Methylation beta values per 1Kb window of the whole genome were calculated by (sum of methylated reads/sum of methylated and unmethylated reads) across all the C bases in the 1Kb window). The beta values were corrected per sample to the lowest conversion rate across the dataset (0.793) through binomial resampling on methylated counts of each region. The beta values were then binned into 5 beta levels (1,2,3,4,5) following: 0–0.2 → 1; 0.2–0.4 → 2; 0.4–0.6 → 3; 0.6–0.8 → 4; 0.8–1 → 5.

The original 1Kb regions across whole genomes went through filtering of the CpGi-rich regions and the regions containing fewer than 3 Cs. The differentially methylated regions (DMRs) were identified through calculating the differences of the beta levels per 1Kb window between each tumor sample and the average of 8 normal esophageal tissue samples (6 from LUD2015-005 and 2 from Barretts2016), and selecting the 1Kb regions with strictly above 90th percentile or below 10th percentile of the beta level differences, and shared by at least 8 samples. All the above resulted in a total of 395,503 1Kb DMR regions identified.

Methylation subtype classification

The matrix of beta levels of 55K randomly selected DMR per sample was used to perform hierarchical clustering of samples (using the hclust method with “ward.D2” linkage). The matrix of beta levels of all 396K DMR per sample was used to perform UMAP embedding ($n_neighbors = 13$). Combining hierarchical clustering and UMAP embedding resulted in five distinct methylation subtypes of OAC identified. The five methylation subtypes were confirmed by repetitive random selection for 100 times of 55K DMR followed by hierarchical clustering and subtype group scoring (for each cycle, all samples grouped within one subtype gained one point for the correspondent subtype).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were done using R. Boxplot visualizations all have a vertical line denoting the minimum to maximum range (excluding outliers), the box marking Q1–Q3, and a horizontal line denoting median. Details for the statistical tests employed can be found in the relevant figure legends. In the case of multiple testing, p -value adjustment was performed using the Benjamini-Hochberg procedure¹¹³ to control for the false discovery rate (FDR). A threshold of 0.05 was used to determine significance throughout, except for DESeq2 differential expression testing, where the package default threshold of FDR <0.10 was used.

ADDITIONAL RESOURCES

The LUD2015-005 study is registered with [ClinicalTrials.gov](https://clinicaltrials.gov) under NCT02735239 (<https://clinicaltrials.gov/ct2/show/NCT02735239>) and with EudraCT under 2015-005298-19 (<https://www.clinicaltrialsregister.eu/ctr-search/trial/2015-005298-19/GB>).