Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods. Supplemental Methods

Eligibility Criteria

This trial was conducted at Columbia University, Cornell University, Washington University, and Brown University. Eligible patients had untreated, histologically-confirmed, locally advanced (≥T2 or lymph node positive) G/GEJ adenocarcinoma, an ECOG PS ≤1, and adequate organ function (absolute neutrophil count ≥1500/μL, hemoglobin ≥9 g/dL, platelets ≥100,000/μL, creatinine clearance ≥60mL/min, total bilirubin ≤1.5X upper limit of normal (ULN), and AST/ALT ≤2.5X ULN). Patients with active infection, immunodeficiency, autoimmune disease requiring therapy within 2 years were excluded.

Study Design and Procedures

At baseline and prior to each treatment, patients were evaluated to ensure treatment parameters were met. CT or PET/CT was performed at baseline, prior to and following surgery, as well as every 12 weeks thereafter. Tumor response was assessed by Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 criteria. Patients were followed for disease progression, recurrence, and survival.

Specimens from baseline biopsy and resection were reviewed locally and independently confirmed for pCR at Columbia University. Pathological response was evaluated independently by the clinical trial pathologist at Columbia University using the College of American Pathologist (CAP) criteria (defined as: 1. Complete response: No viable cancer cells; 2. Near complete response: Single/rare small groups of cancer cells; 3. Partial response: Residual cancer with regression where greater than single/rare small

groups of cancer cells are present; 4. Minimal/poor response: Extensive residual cancer and no evident tumor regression, treatment effect absent) [1].

Toxicity and adverse events were graded according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Up to two dose level reductions per chemotherapeutic agent, with the exception of epirubicin, were permitted. If further toxicity occurred, the responsible agent was discontinued. Chemotherapy was modified per institutional standards. Capecitabine could be shortened to 14 days. Patients who discontinued chemotherapy could continue pembrolizumab. Resumption of pembrolizumab was allowed if improvement in immune-related adverse events (irAE) met protocol-specified criteria.

Statistical Analysis:

Binary and categorical variables are summarized as counts and proportions, and continuous variables as median and range. The primary endpoint of pCR is reported as a proportion along with the one-sided 95% exact binomial confidence interval. The overall response rate (those who achieve CR or PR) is reported for those who had RECIST measurable disease at baseline. DFS was defined from treatment initiation to the date of disease progression, recurrence, or death. Patients who were alive and disease free were censored at last negative scan or the surgery date prior to post-study therapy. OS is defined as the time from treatment initiation to death. DFS and OS were analyzed using the Kaplan-Meier method. DFS and OS analysis was performed in R (version 4.2.1) using the survival package. The risk table for Kaplan-Meier plots was generated using R (version 4.2.1) using the KMunicate package.

Correlative Analyses

As exploratory analysis, we interrogated changes within the tumor immune microenvironment (TIME) with treatment. We compared tumor immune cell infiltration and proximity of tumor cells to immune cells within post-treatment resected samples compared to their respective pre-treatment tumor specimens from 32 patients (31 paired) using quantitative multiplex immunofluorescence (qmIF) with cell-density and proximity algorithms. H&E stained samples were reviewed by two independent pathologists. Specimens were stained for CD8 (clone 4B11; Leica, PA0183), CD3 (clone LN10; Leica,NCL-L-CD3-565), FoxP3 (clone 236A/E7; Abcam, ab20034), PD-L1 (clone 73-10; Leica, PA0832), granzyme B (clone 4E6; LSbio, LS-C338016) and PanCK (clone PCK26; Abcam, ab6401) using Opal™ multiplex kits, and analyzed using the VECTRA® platform and inForm® software as described previously [2-4]. On average, four and six biopsy and resected multispectral images (MSIs) were collected per patient, respectively. A total of 300 MSIs were scanned (113 from biopsy and 187 from resected specimens).

Density of positively stained cells within the epithelial compartment was calculated using a Python script. Cell-cell proximity calculations were performed using the percentage of PanCK+ (epithelial or tumor) cells within a 5μm to 50μm radius of the cell of interest (eg. CD3) and normalized by total PanCK+ cells within each MSI. Average percentages were calculated for each MSI then averaged to the patient level. Mann-Whitney test (two-sided p-values were reported) was used to compare biopsy and resection samples; $p < 0.05$ was considered significant.

eTable 1: Patient Characteristics

Characteristic	N (%)
Age (years)	
Median	65.5 years (25 - 90)
≤ 60	10 (29%)
61 – 70	12 (35%)
> 70	12 (35%)
Sex	
Male	23 (68%)
Female	11 (32%)
ECOG Performance Status (0 – 1)	
0	16 (47%)
1	18 (53%)
Ethnicity	
White	22 (65%)
Asian	5 (14%)
Black or African American	5 (14%)
Unknown	2 (6%)
Tumor Location	
GEJ	13 (38%)
Gastric	21 (62%)
RECIST Measurable Disease at Baseline	16 (44%)
Lymph Node Status (Baseline EUS)	
Node Negative	9 (36%)
Node Positive	16 (64%)
Lauren histologic type (Available)	31
Diffuse type/Mixed	7 (23%)
Intestinal	24 (77%)
Signet Cells (Available)	31
Yes	5 (16%)
No	26 (84%)
Grade (Available)	31
Moderately Differentiated	17 (55%)
Poorly Differentiated	14 (45%)
Baseline PD-L1 (CPS Available)	25
< 1	4 (16%)
≥ 1	21 (84%)
≥ 5	15 (60%)
≥ 10	12 (48%)
MMR/MSI Status (Available)	28
dMMR/MSI-H	3 (11%)

Abbreviations:
ECOG, Eastern Cooperative Oncology Group; GEJ, Gastroesophageal Junction; RECIST, Response Evaluation Criteria in Solid Tumors; EUS, endoscopic ultrasound; PD-L1, programmed death-ligand 1; MMR, mismatch repair; MSS, microsatellite stable; MSI, microsatellite instable. *Includes two patients with PD-L1 CPS scores >1.

eTable 2: Pathological Responses

Pathological Response (Central Review)	Evaluable N=34 (%)	Curative Resection: N=28 (%)
Complete (pCR)	7 (20.6%)	7 (25%)
Near-complete (pNCR)	6 (17.6%)	6 (21%)
Partial (pPR)	8 (23.5%)	8 (29%)
Treatment effect present, NOS	1 (2.9%)	1 (4%)
No or minimal/poor (pMR)	7 (20.6%)	7 (25%)

Abbreviations:

PCR, pathological complete response; pNCR, pathological near-complete response; pPR, pathological partial response; NOS, not otherwise specified; pMR, pathological minimal response.

eTable 3: Adverse Events

	Related to Any Treatment (Patients with maximum grade)				Related to Pembrolizumab	
Adverse Event	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 3**	**Grade 4**
Any Type	4 (11.4%)	10 (28.6%)	15 (42.9%)	3(8.57%)	9 (25.7%)	1 (2.9%)
Diarrhea	15 (42.9%)	4 (11.4%)	7 (20.0%)	0(0%)	6 (17.1%)	0 (0%)
Anorexia	5 (14.3%)	3 (8.6%)	4 (11.4%)	0(0%)	0 (0%)	0 (0%)
Anemia	3 (8.6%)	3 (8.6%)	3 (8.6%)	0(0%)	0 (0%)	0 (0%)
Neutropenia	1 (2.9%)	6 (17.1%)	3 (8.6%)	0(0%)	0 (0%)	0 (0%)
Dehydration	1 (2.9%)	0 (0%)	1 (2.9%)	1(2.86%)	0 (0%)	0 (0%)
Fatigue	16 (45.7%)	5 (14.3%)	2 (5.7%)	0(0%)	1 (2.9%)	0 (0%)
Febrile neutropenia	0 (0%)	0 (0%)	0 (0%)	2(5.71%)	0 (0%)	0 (0%)
Hypokalemia	1 (2.9%)	0 (0%)	1 (2.9%)	1(2.86%)	0 (0%)	0 (0%)
Hyponatremia	0 (0%)	0 (0%)	2 (5.7%)	0(0%)	2 (5.7%)	0 (0%)
Sepsis	0 (0%)	0 (0%)	0 (0%)	2(5.71%)	0 (0%)	1 (2.9%)

The most common (>5%) TRAEs are indicated

eTable 4: Treatment Completion

Treatment Tolerance of Evaluable Patients (N = 34)	N (%)
Initiated neoadjuvant treatment	**34 (100%)**
Pembrolizumab with:	
Epirubicin, Oxaliplatin, and Capecitabine	**2 (6%)**
Capecitabine and Oxaliplatin	**32 (94%)**
Neoadjuvant Therapy (Cycles 1 – 4)	
Completed all allocated therapy	**27 (79%)**
Chemotherapy (Cycles 1 – 3)	**29 (85%)**
Pembrolizumab (Cycles 1 – 4)	**29 (85%)**
Adjuvant Therapy (Cycles 5 – 7)	
Started all allocated therapy	**15 (44%)**
Maintenance Therapy (Cycles 8 – 21)	
Completed at least 17 cycles	**13 (38%)**

eResults. Supplemental Results

Safety

Treatment-related adverse events (TRAEs) and immune-related adverse events (irAEs) were reported in 34 and 28 patients, respectively (**eTable 3**). The most common (>5%) grade ≥3 TRAEs are indicated (**eTable 3**). Twenty-seven (79.4%) patients completed all allocated neoadjuvant therapy, permitting dose-holds and modifications.

Treatment tolerability

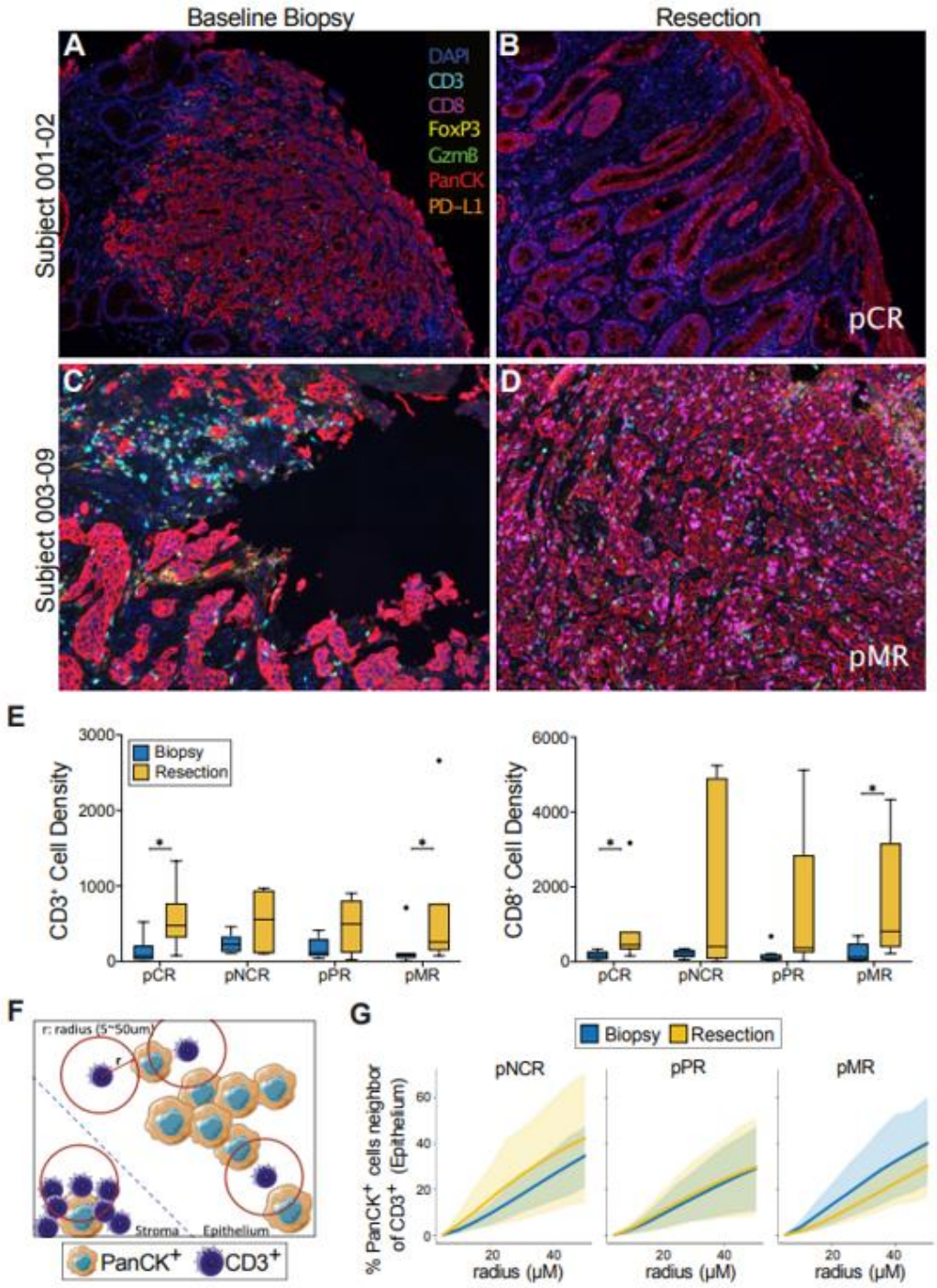
Only two (6%) patients also received epirubicin (**eTable 4**). Two patients discontinued all treatment (one due to physician discretion and another due to grade 3 irAE (rash) and grade 3 AE (diarrhea) from capecitabine). One patient stopped pembrolizumab after 3 cycles due to grade 3 irAE from hemorrhagic colitis and one patient was unable to complete neoadjuvant oxaliplatin due to an infusion reaction. Twenty-nine patients underwent surgical resection, 28 (82%) with curative intent (**Figure 1**). Fifteen (44%) patients started all allocated adjuvant therapy and at the data cut off, 13 (38%) had completed at least 17 cycles of maintenance pembrolizumab.

Exploratory Analyses

To explore changes within the tumor microenvironment (TME) following COP, resection samples were compared to baseline biopsies using quantitative multiplex immunofluorescence (qmIF). Paired specimens from 31 patients were stained for DAPI (nuclear), CD3 (pan T-cell), CD8 (cytotoxic T-cell), Granzyme B (activation), FOXP3 (T-

regulatory lymphocyte), PD-L1, and PanCK (tumor and/or epithelial cells). Representative paired biopsy and resection images from a patient who achieved a pCR (001-02) and a patient who had a minimal response (pMR) (003-09) to therapy are depicted in **eFigure 1A-D** (**Supplement 2**). We observed increased $CD3^+$ and $CD8^+$ T-cell densities within resected as compared to tumor biopsy specimens regardless of degree of pathological response (**eFigure 1E** in **Supplement 2**). To determine the effect of therapy on intercellular distances and whether these changes correlated with degree of pathologic response, cell-cell proximity analysis was performed. The percentage of $PanCK^+$ cells within a range of distances of $CD3^+$ cells was calculated (**eFigure 1F** in **Supplement 2**). Compared to baseline, clustering of $PanCK^+$ cells to $CD3^+$ cells within post-treatment samples increased with the degree of pathological response (**eFigure 1G** in **Supplement 2**).

eFigure. Changes in the Tumor Immune Microenvironment



Evaluation of changes to the tumor immune microenvironment following treatment with COP. A-D. Representative quantitative immunofluorescence images from baseline and resection samples obtained from a patient who achieved a pathologic complete response (pCR) (A & B)

and a patient who had pathologic minimal response (pMR) (C & D). **E.** Density of CD3+ (left) and CD8+ (right) T-cells in baseline biopsy and resected samples grouped by degree of pathological response. Asterisk (*) indicates $p < 0.05$, Mann-Whitney test. **F.** An illustration of near neighbor analysis for PanCK+ cells near CD3+ T-cell (cell of interest) over a distance of 5-50μM is shown. **G.** The percentage of PanCK+ cells within a range of distances of CD3+ cells grouped by degree of pathological response are shown. Abbreviations: PCR, pathological complete response; pNCR, pathological near-complete response; pPR, pathological partial response; NOS, not otherwise specified; pMR, pathological minimal response.

eReferences

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